Developing novel systems for expression of intracellular membrane-bound human cytochrome P450 enzymes in baker's yeast

by

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Abstract

Human cytochrome P450 (CYP) enzymes belong to a family of monooxygenase enzymes that are responsible for the metabolism, in a stereo-specific and stereo-selective manner, of diverse chemicals. These chemicals are often referred to as xenobiotics which are usually harmful and toxic to the human organism. CYP enzymes play a key role in the metabolism of pharmaceutical drugs, making them more soluble for excretion from the human body. Human CYPs are also naturally involved in the biosynthesis of vitamins, steroids, fatty acids, lipids and cholesterol.

A CYP enzyme requires a CYP reductase (CPR) for it to be active. Both CYPs and CPR are bound to the intracellular endoplasmic reticular (ER) membranes. The presence of a CPR is absolutely essential for membrane-bound CYP activity. It seems another ER-bound protein, cytochrome b5, is required for the activity of some CYPs. However, cytochrome b5's role in CYP activity is not clearly understood.

Drug metabolism research in pharmaceutical industry involves accurately (a) determining the potential of a drug and its metabolites to inhibit CYP enzymes and (b) identifying its metabolites which are formed through CYP-mediated biotransformation reactions. The degree of success in drug metabolism research is a crucial measure for approval/rejection of a drug by regulatory authorities. However, drug metabolism studies take place only in the pre-clinical – clinical interface which is quite late in the drug discovery process for a failure to occur, which unfortunately happens quite often. Drug metabolism studies are not performed any earlier primarily because CYP enzymes are extremely costly and are difficult to use because of their inherent instability at room temperature. The experiments performed in this thesis attempt to address these issues. The main aim of the work was to

create a new set of tools that would facilitate the drug discovery process in general but, more specifically, allow these reagents to be used by chemists/biologists in early pre-clinical phase, where they are most needed, by trying to overcome the major hurdles of CYP's costs and stability.

There are five experimental Chapters (Chapters 3 to 7) of this thesis.

Chapter 3 describes the expression of 17 human CYP enzymes in baker's yeast, using human CYP genes chemically synthesized with yeast-biased codons. There are 61 codons which code for the 20 amino acids that are essential for protein syntheses. It is known that the highly expressed genes, in a particular organism, use specific codons which would indicate that there is codon bias. Hence, codons used by highly expressed genes in baker's yeast were used to create genetic sequences for human CYP enzymes. These codon-optimised human CYP genes, in theory, should provide high expression in yeast. Therefore, expression of synthetic genes, from episomal (i.e. 'extra-chromosomal') plasmids, was compared with the expression of native genes, isolated from a human liver cDNA library to assess the assumption that yeast-biased codons would provide better expression of human CYPs. It was clearly shown that, per constant number of yeast cells, the synthetic genes expressed far more CYP activity than the native genes. It was also shown that some CYPs which have been claimed to require cytochrome b5 for their activity may not require its presence.

Chapter 4 describes the expression of one copy of synthetic genes (chemically synthesized with yeast-biased codons), from a single yeast 'chromosomal' locus to find out which locus gives the best expression of human CYP proteins. Expression of genes from chromosomal loci allows growth of yeast cells in cheap, 'non-selective' growth

media, continuously over 5 days, or longer, in shake-flasks or fermentors. In contrast, expression from extra-chromosomal, episomal plasmids demand growth of yeast cells in 'selective' growth media, which are expensive, where cell numbers are relatively low, and cell growth is restricted to 24 h. It has been speculated that, heterologous (i.e. foreign) gene expression from yeast depends on the yeast proteins that reside in the neighbourhood of the human protein that is being expressed from a particular yeast chromosome. Hence, CYP gene expression cassettes (consisting of a promoter, the gene of interest and a transcription terminator) were integrated into different chromosomal loci, using homologous recombination, a technology which also facilitates gene therapy in human cells. The results obtained clearly show that there is differential expression of a human CYP enzyme when expressed from the neighbourhood of the yeast ADE2, HIS3 and URA3 genes. Indeed, the best human CYP expression occurs from a particular locus of chromosome XV, where the yeast HIS3 gene resides.

Chapter 5 is a continuation of Chapter 4 where two copies of chromosomally integrated human CYP genes (chemically synthesized with yeast-biased codons and integrated at two different chromosomal loci, via homologous recombination), have been used to express human CYPs from baker's yeast. The goal was to obtain high yields and activities of human CYP enzymes. In order to achieve this, a new process was developed for isolation of microsomal [i.e. endoplasmic reticular (ER) membrane bound] enzymes. CYPs are totally inactive when shorn off these ER membranes. The activities of the baker's yeast produced human CYP enzymes have been compared, head-to-head, with the three commercially available enzymes which are produced either from insect cells or bacterial cells and which are sold worldwide. Global market size of recombinant CYPs is considered to be at least $250 million. The results obtained show that the human CYP

enzymes, produced from baker's yeast, are much more active than the commercially available enzymes. The results also suggest that, without embarking on production of these enzymes in a cheaper environment, the human CYP enzymes could easily be produced in the UK and sold at half the price quoted by the current manufacturers. Yet these cheap, highly active enzymes, if marketed, could still provide a profitable margin. Because of their cheapness, they could be made widely available for early pre-clinical research.

Chapter 6 explores the use of three CYP-producing recombinant cells for biotransformation reactions. It has been reported that, until now, whole cell mediated biotransformation reactions generally yield, at best, no more than 10-15% of the product. Whole recombinant yeast cells, expressing (a) CYP1A1, (b) variants of human CYP2D6 and (b) human CYP3A4 have been used to transform (i) chrysin, a natural product, (ii) codeine, a CYP2D6 substrate, and (ii) the CYP3A4 substrate, AZD-2014, an Astra-Zeneca drug in multicentre Phase II/Phase III clinical trials. The results from LC/MS, HPLC and TLCs clearly show that there is >80% product formation. They also indicate that, in future, organic chemists in pre-clinical drug discovery could use this type of robust whole yeast cells, harbouring human CYPs, for bioorganic reactions.

Chapter 7 describes the successful simultaneous co-production of two or three different human CYPs within the same cells. It is likely that two specific CYPs function, in tandem, on a substrate. There is also a necessity of determining inhibition of a particular human CYP, in the presence of another CYP or other CYPs. It is believed that the inhibition of a CYP is likely to be altered in the presence of another CYP through a biochemical process known as the 'crowding effect'. These hypotheses can, in future, be tested using

the recombinant cells reported in this Chapter. The cells, co-expressing two or three human CYPs, could also be used for both biotransformation reactions and isolation of microsomal enzymes. The concept of co-expression of CYPs could also be used, in the future, as a stepping stone for creation of liver-like yeast cells which have the ability to express multiple CYPs of choice.

In summary, this thesis describes various yeast systems that have been created for efficient Drug Metabolism studies and Biotransformation reactions.

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Abbreviations

BSA	Bovine Serum Albumin
bp	base pair
cDNA	Complementary deoxyribonucleic acid
CO	Carbon Monoxide
CPR	NADPH-P450 reductase
CYP	Cytochrome P450
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylene diamine tetra acetic acid
ER	Endoplasmic reticulum
G6P	Glucose-6-phosphate disodium salt hydrate
G6DPH	Glucose-6-phosphate dehydrogenase
H2DCFDA	5-(and-6)-carboxy-2´,7´-dichlorodihydrofluorescein diacetate
hRD	Human NADPH P450 reductase
IC_{50}	The half maximal inhibitory concentration
kbp	kilobase pair
LB	Luria-Bertani (medium)
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
$NADP^+$	β-Nicotinamide adenine dinucleotide phosphate
NADPH	β-Nicotinamide adenine dinucleotide phosphate (reduced form)
OD	Optical density

PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
RFU	Relative fluorescence units
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium Dodecyl Sulfate
TAE	Tris/EDTA/Glacial acetic acid buffer
TE	Tris-HCl/EDTA
TEMED	*N*,*N*,*N'*,*N'*,-tetramethylethylenediamine
Tris-HCl	Tris(hydroxymethyl)aminomethane-HCl
U	Unit
UV	Ultraviolet
V	Volt
yRD	Yeast NADPH P450 reductase
YIp	Yeast integrative plasmid
YEp	Yeast episomal plasmid
YRp	Yeast replicative plasmid
YAC	Yeast artificial chromosomes
HIS3	Histidine locus 3
URA3	Uracil locus 3
ADE2	Adenosine locus 2
TRP1	Tryptophan locus 1

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CHAPTER 1 Introduction

1.1 Background

Every single living creature is exposed to different chemicals. They are known as xenobiotics. A xenobiotic is a chemical not produced by the human body ors such, a living organism. Essentially, it is foreign to an organism. After assimilation of these hazardous chemicals into the human body, the absorbed xenobiotics are eliminated from the body effortlessly through bodily liquids like the sweat, urine or faeces. However, the xenobiotics that are fat-soluble can build up in the lipid-rich tissues which have no mechanism to remove them. This could be lethal to the body. The conversion of xenobiotic chemicals to soluble substances helps the body to fight against their accumulation and, at the end, avoid any harm. The process of conversion (i.e. metabolism) includes transformation of xenobiotics into more polar (i.e. hydrophilic) substances which encourages their expulsion from the body, a process known as detoxification (Masters 1998).

The detoxification process happens in three stages. Xenobiotics undergo solubilisation via biotransformation reactions directed by Phase I, II and III metabolising enzymes. The Phase I enzymes are involved in chemical reactions that catalyse oxidation, reduction or hydroxylation. Usually functional groups, such as –OH, –NH2, or –COOH, are added to the parent compound (i.e. the xenobiotic) to make it more polar. Mono-oxygenation reactions that allow hydroxylation are the most common amongst the Phase I enzymes. Hydroxylation involves addition of –OH group(s) to unreactive carbon atoms. They are catalysed by cytochrome P450 (CYP) enzymes. Phase II detoxification includes

conjugation with a small endogenous substance like glutathione, glucuronic acid, or cysteine. Responses in Phase II make the metabolites, obtained from reactions in Phase I, considerably more hydrophilic and help in their removal by means of bile or urine. Usually, Phase II metabolism further diminishes the pharmacological activity of the metabolite obtained in Phase I. Phase III reactions use products gotten from Phase II conjugation reactions. These are catalysed by similar enzymes which are dynamic both in Phase I and II metabolism (Danielson 2002) .

Chemical Reactions Involved in different stages of xenobiotic metabolism:

- Phase I – Oxidation, reduction and hydrolysis,
- Phase II – Conjugation,
- Phase III – Oxidation, hydrolysis and deamination.

1.2 The birth of CYP450 (CYP) Enzymes

The existence of cytochrome P450 (a CYP) was first recognized by Martin Klingenberg, (Klingenberg 1958) who was studying the spectrophotometric properties of pigments in a microsomal fraction prepared from rat livers. The term ‘cytochrome P450’ was coined in 1962, what was thought to be at that time, as a temporary name for a coloured substance in the cell (Mason 1957, Cooper et al. 1965) . This pigment, when reduced and bound with carbon monoxide (CO), produced an unusual absorption peak at a wavelength of 450 nm. Cytochrome is a misnomer given that a CYP is an enzyme rather than a true cytochrome. Despite this, the name ‘cytochrome P450’ has remained and is now widely accepted. At first, it was believed that there was only a single CYP enzyme. However,

today human and other mammals are known to have more than 50 distinct CYP enzymes. The total number present in plants is much more than 50, often as high as several hundred (usually 400-500). Over the last 15 years, CYP research has largely been concerned with defining the multiplicity of CYP enzymes in humans and diverse ranges of organisms. In more recent years, with CYP multiplicity having largely been determined, functional studies with CYP enzymes have begun to take precedence.

1.3 Naming of CYP450 Enzymes

Over 1000 different CYPs have partially been characterized in different organisms of the biological kingdom. A standardized nomenclature is used to describe the different CYPs that have been identified (Nelson *et al*., 1996). A P450 enzyme is denoted by the acronym "CYP" (cytochrome P450), an Arabic number denoting the family, a letter standing for the subfamily followed by an Arabic numeral that represents individual enzymes in the subfamily. This nomenclature does not indicate any function of a CYP family member.

There are at least 12 CYP gene families found in humans. Three of these families are involved in the metabolism of important drugs (i.e. pharmaceuticals), mainly through hydroxylation, which allows excretion of soluble metabolites of the drugs from the body. The three CYP families involved in most drug metabolism reactions are CYP1, CYP2 and CYP3 (Zanger, Schwab 2013) . The family members are identified by the amino acid sequences that they have in common. As mentioned earlier, each family consists of sub-families. Uppercase letters (e.g. 1B, 2C, 3A, 2B) represent the subfamily and each enzyme can be further differentiated by Arabic numerals (e.g. 3A4, 2B6, etc.). CYP3A4 is the

most abundant CYP enzyme in humans. 30% of all CYP enzymes in the liver and 70% of those in the gut consist of CYP3A4.

As industrial-scale technologies have evolved, organisms including humans have continued to be more and more exposed to dangerous chemicals collectively known as xenobiotics (Bezirtzoglou 2012) . Xenobiotics are found everywhere. They could consist of chemicals which are pollutants, pesticides, medicines, plant alkaloids and toxins, all of which human beings have to imbibe during their day-to-day existence. Most of these xenobiotics are easily absorbed, due to their lipophilic nature, into the human body via the skin, lungs and intestines. If absorbed and not metabolised, they become toxic. A xenobiotic has to be converted into a more water-soluble compound before it is excreted.

The Phase I and Phase II enzymes that are responsible for the metabolism of xenobiotic compounds have been described by (McClellan, Brodsky 2000) ; Table 1.1.

Table 1.1. Reaction and groups of enzymes involved in the metabolism of pharmaceutical drug substances.

Class of Reaction	Phase I or II	Enzymatic Activities
Oxidation	Phase I	CYP450 mediated, Alcohol dehydrogenase, Aldehyde dehydrogenase, Xanthine oxidase, Monoamine oxidase, Flavin mono-oxidase
Reduction		Quinone reduction, Reductive dehalogenation
Hydrolysis		Epoxide hydrolase
Glucuronide conjugation	Phase II	UDP-glucuronosyltransferase
Gluthathione conjugation		Glutathione S-transferase
Sulphate conjugation		Sulfotransferase
Acetylation		N-acetyltransferase
Methylation		Methyltransferase

In 1956, JK Grant discovered that in mammalian steroidal hydroxylase systems the electron donor is NADPH. Later, it was found that the enzymes that performed steroid hydroxylation and which are also involved in cholesterol metabolism are proteins that belong to the cytochrome P450 (CYP) family. Some of them are associated with the mitochondria while others to the endoplasmic reticulum, which are intracellular compartments of a eukaryotic cell. Their activities are all supported by NADPH and two other electron-transfer proteins (Manson 1957) .

In 1957, Manson introduced the term mixed-function oxidase for oxygenase reactions in which the substrate, oxygen and electron donor come together in a tripartite reaction to produce (a) a hydroxylated substrate, (b) the oxidised form of the electron donor and (c) water.

In sterol metabolism, hydroxylase enzymes perform irreversible reactions, which often results in the conversion of a ubiquitous sterol substrate into a metabolite with selective and potent properties. Microsomal (i.e. endoplasmic reticular) fraction of certain cells is where the biosynthesis of cholesterol occurs through the anaerobic reaction between acetate and squalene. The reaction requires the involvement of oxygen and NADPH (Van Tamelen et al. 1966, Cory, Russey & Ortiz de Montellano, P R 1966) and is catalysed by a CYP450 enzyme. This result seems to be in conflict with the data published by (Shozo Yamamoto, Konrad Bloch 1970) where they claimed that epoxidation, mediated by an enzyme of the CYP family, was not inhibited by carbon monoxide (CO). CYPs ought to be inactivated by CO since coordination (binding) of CO to ferrous haem iron de-activates its hydroxylase activity.

Bergstrom, in 1959, provided more evidence for the involvement of CYPs in sterol biosynthesis showing that one route for the conversion of cholesterol into bile acids involves the hydroxylation of cholesterol to 7α-hydroxy-cholesterol. This process occurs mainly in the liver microsomal fractions, the hydroxylation reaction again requiring NADPH and oxygen. Although the reaction, which is now known to be mediated by a CYP, was inhibited by carbon monoxide, the inhibition was shown to be reversed by light at 450 nm (Scholan, Boyd 1968) .

In 1995, Mason, Fowlks and Peterson published that in specific enzymatic hydroxylation reactions mediated by diverse hydroxylases, molecular oxygen donates an oxygen atom that is transferred to the substrate. These hydroxylases formed the basis of the cytochrome P450 (CYP) family of enzymes.

1.3.1 Cytochrome P450 (CYP) enzymes in humans

Although there are a number of oxidative enzymes capable of undertaking xenobiotic metabolism (e.g. alcohol dehydrogenase, xanthine oxidase and the amine oxidases), the main oxidative enzyme system in xenobiotic metabolism involves the family of cytochrome P450 (CYP) enzymes. The CYP enzymes are haem-containing membrane proteins that metabolize both foreign chemicals (xenobiotics) and endogenous compounds. CYPs are known to act in humans as drug metabolizing enzymes, but in human cells the primary purpose of these enzymes is to make essential chemicals, such as steroids (i.e. cholesterol) and other important lipids such as prostacyclins and thromboxaneA2 (Daum et al. 1999) . It has been found that some CYPs are intimately involved in vascular autoregulation, particularly in the brain (Fleming 2001) . Mutations in cytochrome P450 genes or deficiencies at the enzyme levels are responsible for several human diseases. Some CYPs may activate pro-carcinogens to carcinogens (Code 1997; Su 2000); many are also involved in the removal of carcinogens from the body(Gonzalez, Kimura 2003) ; and reports indicate that certain CYPs are involved in the promotion of lung cancer in cigarette smokers(Antariksa Budhi 2003) .

Most of the known CYPs are found at different constitutive levels in the liver of the human body (Nelson et al. 1996) , which is the main organ involved in drug and toxin removal. However, substantial amounts of CYPs are also found in extrahepatic tissues

such as, the small intestine, the lungs(Simon D. Spivack et al. 2001) , kidney, skin, heart and brain(Gonzalez, Kimura 2003, Rieder et al. 2000) . In the human liver there are at least 12 distinct CYP enzymes. At present it appears that only six isoenzymes from families CYP 1, 2 and 3 are involved in hepatic metabolism of most of the marketed pharmaceutical drugs. They are CYP1A2, CYP3A4, CYP2C9, CYP2C19, CYP2D6 and CYP2E1. These CYPs account for about 70% of the human hepatic microsomal CYPs, with CYP3 isoenzymes accounting for approximately 30%. The major pharmaceutical drugs that they metabolise (R H Levy 1995) are listed in Table 1.3.

1.3.2 Mechanism of action of CYP450 enzymes

There are two subunits which are required for the activity of human cytochrome P450 (CYP) enzymes. They are the haem containing catalytic subunit, cytochrome P450 (CYP), and the activating subunit which consists of NADPH-dependent P450 reductase (CPR). NADPH is the reducing agent which reduces atmospheric oxygen (O_2), inserting an atom of oxygen into the CYP substrate (which could be a potential medicine), with the other oxygen atom being reduced to water. The stoichiometry of the reaction is depicted below (White, Coon 1980) :

$$RH + NAD(P)H + H^+ + O_2 \rightarrow ROH + NAD(P)^+ + H_2O$$

1.3.3 NADPH-cytochrome P450 reductase (CPR)

CPR, a flavo protein, is situated on the endoplasmic reticulum (ER), functioning as a redox partner to a CYP also embedded on the ER, supplying electrons necessary to

catalyse CYP-dependent oxygenations. In some cases, CPR may have a conformational role on a CYP, affecting the latter's function by changing a step in the reaction cycle which is not involved in electron transfer (open and close conformational changes). When it is in close conformational form, the electron passes through FAD and then to FMN before it gets to the haem-protein because FMN-binding site is covered by the FAD binding domain whereas if they are in open conformation (close proximity), there will be a direct passage of the electron from NADPH to FMN and to the haem-protein (Guiterrez et al., 2002).

The crystallographic structure of CPR suggests that this protein may have emerged from gene fusion. CPR is one of only four mammalian proteins known to contain both FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide) domains in a single polypeptide chain. The simplest system that supports NADPH-dependent oxygenation reaction *in vitro* involves the three major components, namely,

(a) A CYP450 (CYP) catalytic subunit,

(b) The NADPH-cytochrome P450 reductase (CPR), and

(c) A phospholipid (Lu, Junk & Coon 1969, Lu, Coon 1968, Henry W. Strobel et al. 1970) .

The reaction between the CPR and CYP is required to ensure that electrons from NADPH passes through CPR to the CYP in two single-electron transfer steps (Figure 1.1; (White, Coon 1980, Loida, Sligar 1993) .

There is another protein that contributes to the monooxygenase function of CYP450s (CYPs). It is the cytochrome b5. The b5 protein can serve as an allosteric effector of CYP

activity without supplying electrons for CYP-dependent substrate metabolism (Hiroyuki Yamazaki et al. 1996, Porter 2002) .

There is only one CPR that exists in humans whereas in plants there are multiple forms of the enzyme.

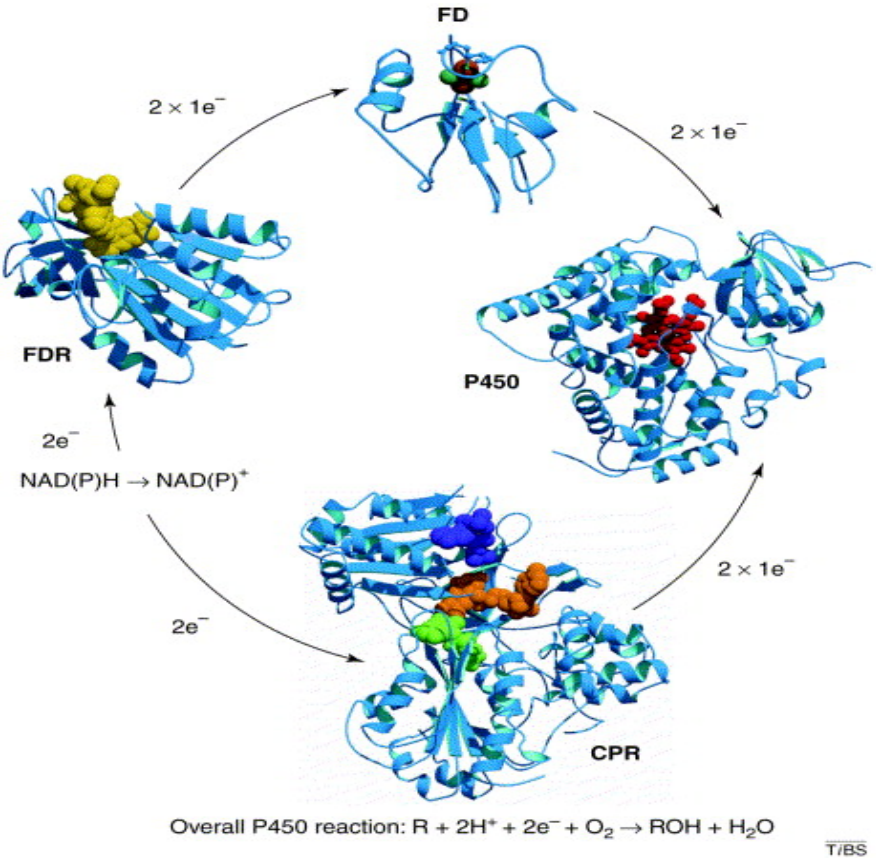


Figure 1.1. Cytochrome P450 redox system. P450s receive electrons by one of two major routes for oxygenation of substrate (R). Most P450s generate hydroxylated product (ROH). The two electrons are delivered through NADPH. In the class I P450 system (e.g. bacterial and mammalian adrenal mitochondria), the electrons are shuttled through flavin adenine dinucleotide (FAD), via ferredoxin reductase (FDR) and a ferredoxin (FD), to the P450. In the class II P450 system, (e.g. mammalian hepatic drug-metabolising isoforms) electrons are delivered by a FAD and Flavin mononucleotide (FMN)-containing P450 reductase (NADPH-cytochrome P450 reductase (CPR) to the P450.(HEME in red, NADP+ in dark blue , FAD in yellow, FAD in orange and FMN in green). (Adapted from (Andrew W Munro et al. 2002) **; Trends in Biochemical Sciences).**

1.3.4 The role of cytochrome b5 in cytochrome P450 (CYP) mediated reactions

Cytochrome b5 has a role in electron donation to microsomal desaturases that are involved in the synthesis of unsaturated fatty acids (Vergères, Waskell 1995) . It is a 17-kDa haem-protein present in the endoplasmic reticulum (ER) of all eukaryotic cells. Together with NADPH, it helps in CYP450 monooxygenase reactions. Recent studies have demonstrated that in the expression of some recombinant CYP enzymes (CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2E1, CYP4F3, CYP4F2, CYP2J2, CYP4A11 and CYP3A4) b5 is required for maximal catalytic activities. In the same study, it was also suggested that the condition for reconstitution of activities vary depending on the CYP450 enzymes that are expressed (Hiroshi Yamazaki, Tsutomu Shimada, 2006). This study has shown that:

- CYP2C8, CYP2C9, CYP2C19, CYP4F3, CYP4F2, CYP2J2, CYP4A11, CYP2E1 and CYP3A4 have increased activities when cytochrome b5 (b5) was co-expressed along with the CYP450 reductase (CPR), and
- Although CYP2C19 is closely analogous to CYP2C18 in the protein sequence, the latter may not require cytochrome *b5* to manifest activity.

In the past, it has been reported that CYP3A4 co-expressed with b5 reductase and b5 produce greater rates of reaction than when CYP3A4 is co-expressed just with CYP450 reductase (CPR) (Figure 1.2; Yamazaki *et al*). In 1971, Hildebrandt and Estabrook established that stimulation of P450 drug oxidations with the addition of NADH to microsomes shows that cytochrome b5 contributes the second electron to the CYP450

catalytic cycle. Activity of CYP2B4 inhibited by cytochrome b5 at higher concentration which suggested that cytochrome b5 was occupying the cytochrome P450 reductase-binding site on cytochrome P450 2B4 (Haoming et al., 2008). However, in the studies reported here, I was able to show that not all CYPs require this extra electron from cytochrome b5.

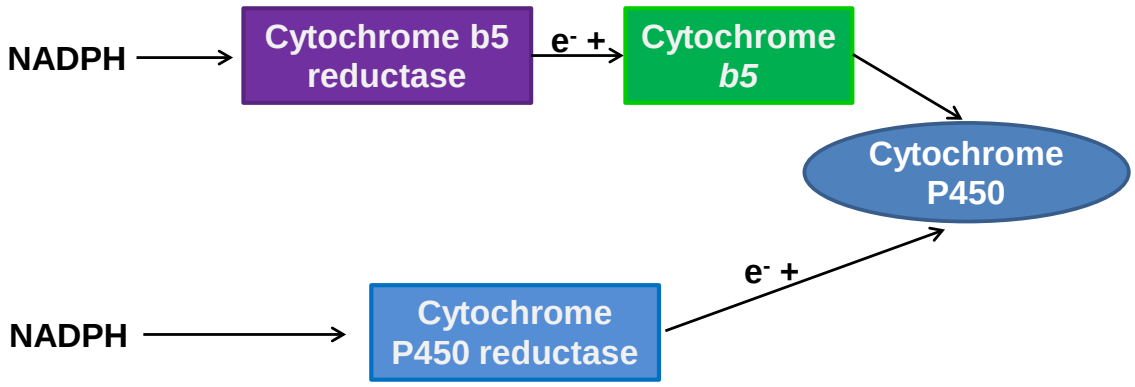


Figure 1.2. The electron pathway transport system of cytochrome P450 in the endoplasmic reticulum. The cytochrome b5 is readily reduced upon addition of NADPH, and that cytochrome reductases (purple and light blue colours) serve as electron donors to both cytochrome b5 (green colour) and the cytochrome P450 (deep blue). The cytochrome b5 serves as an additional electron donor to P450-mediated reaction. (Adapted from Todd D. Porter, 2002)

1.4 Stress Response, Stress Genes and Protein Expression

There are different conditions that cells are subjected to during cell division. These conditions prevent cells to produce maximum amounts of cellular proteins when required. Increase of temperature, increase or decrease in pH, salinity or oxygen concentration could be stressful for cells. The result of cellular stress can lead to protein

denaturation which involves proteins losing their native, functional configuration and ultimately leading to aggregation.

This process is likely to be reversible to some extent but cells die when they suffer from stress beyond a certain limit. Another factor that prevents cells from producing more cellular protein is the down-regulation of the house keeping genes. This machinery may be shut off or inactive due to the nature of proteins that are being over-expressed or over-produced.

Part of the response towards cellular stress is the activation of stress genes leading to higher expression of stress proteins. These stress proteins protect cells from the effect of the protein denaturation and help cells to recover from the effects of denatured cellular proteins. Most of the stress proteins include heat shock proteins (HSPs) but also several others which function similarly to protect cells. Most stress proteins act in the absence of stress, under normal conditions. They play roles in

a) Protein biogenesis,

b) Assisting correct folding of proteins,

c) Metabolism and metabolite transport

d) Aiding translocation of proteins across membranes of intracellular compartments (organelles) guiding to places where they live and function, and

e) Supporting assembly of protein complexes allowing them to acquire multi-subunit structures for manifestation of activity ((Alberto J. L. Macario et al. 1999)).

1.5 Codon Bias and Heterologous Protein Expression

Genentech scientists and their collaborators (1977) produced the first recombinant human protein (somatostatin) in a bacterium. They were the first who were able to express a human protein in a heterologous host which played a vital role in the development of future biotechnology industries. With only the amino acid sequence available, the group was able to synthesize 14 codon long somatostatin, using oligonucleotides, instead of cloning it from the human genome. In the absence of technology for constructing cDNA libraries, it was the only way to clone a gene in those days. But now, genes are cloned from tissue (or organism)-specific cDNA libraries directly using gene-specific primers and the polymerase chain reaction (PCR).

There are 61 nucleotide triplets (codons) that code for 20 essential amino acids. There are three more which are nonsense codons, also known as stop codons, which help to end gene transcription. An amino acid can be coded by a single triplet (codon) as in the case of Methionine (Met, M) and Trytophan (Trp, W) or it could be coded by up to six codons as in the case of Arginine (Arg, R), Leucine (Leu, L) and Serine (Ser, S).

The degeneracy of the genetic code enables alternative nucleic acid sequences to code for the same protein. The usage of specific codons varies

- From one organism to the other,
- Between proteins highly expressed and at low levels within the same organism,
- Often many times within the same operon, and

- In response to GC content of the gene.

The level of gene expression depends on various factors such as

- Gene copy number, transcriptional control elements,
- The site of chromosomal integration,
- mRNA stability, and
- Translational efficiency.

Several efforts have been made to improve the level of protein expression in mammalian cells. They have concentrated on the elements involved in the gene copy number and transcription/translation determining elements.

Gross and Hauser (1995) have shown that translational events play a vital role in limiting the expression of a gene. Protein coding system for gene expression can be adjusted by manipulating the vector construction, gene transfer method and selection protocol. In contrast, the control of genes at the translational levels is controlled by coding gene structure. Although the mechanisms controlling this type of gene regulation are still unknown, there has been some success towards understanding by introducing

(a) an intron sequence that directs the pre-mRNA into the splicing pathway (Petitclerc et al. 1995) , and

(b) the sequences that facilitate mRNA such as a Kozak consensus sequence (Kozak 1987) .

Kim *et al.* (1997) suggested that comparison of performance of genes re-engineered with either human or yeast favoured codons will provide more useful information about the factors affecting gene expression with respect to codon usage and mRNA secondary structure. Experimental evidence showed that the highest protein expression was obtained with 'hybrid' codon usage where part of the genetic sequence was optimised for codons specific for the organism in which the protein was being expressed.

1.6 Expression of human genes synthesised using yeast biased codons

Codon usage bias is a phenomenon in which synonymous codons encoding the same amino acids are used at different frequencies. It has been suggested that codon distribution often occurs due to mutations or natural selection equilibrium between similar codons in each organism.

In this report, I will be describing experiments which lead to the production of CYP enzymes that could be used

(a) for drug metabolism studies, and/or

(b) as potential targets for the treatment of breast cancer and prostate cancer.

We have chosen to use yeast biased codons to recreate sequences of different human cytochrome P450 (CYP) genes. The codons used are utilized by highly expressed genes in baker's yeast. The aim was to find out the consequence on CYP protein expression levels when chemically synthesised human *CYP* genes, using 'yeast biased' codons, were expressed in yeast. The conjecture was that, perhaps the human *CYP* genes would be

expressed more at the mRNA level than the genes isolated from a human cDNA library. This may result in higher protein levels. The concept relies on more 'stable' transcription, that is, if CYP mRNAs are transcribed in a way that the yeast cells do not recognize the human genes as foreign, there should be more human CYP proteins produced in yeast cells provided there is no block during translation. CYP activities could then be monitored. Higher CYP activities could be assumed to correlate with higher protein production.

1.7 Role of CYP450s in Drug Metabolism

Drug metabolism is a major determinant of pharmacokinetics, a process that regulates drug action. The vast majority of small molecules and biotechnological drugs are metabolized while some others are excreted as unchanged parent drug. Drug metabolism plays a very important role in the inter-individual differences in drug response and also drug interactions which may result in adverse effects leading to the failure of a therapeutic drug.

Most clinically used drugs are chemicals which do not belong to the normal composition of the human body. Hence, they are also referred to as xenobiotics. The principle of elimination of drugs that are foreign substances from the body is through metabolism, a form of biotransformation. As described earlier, this elimination occurs via Phase 1 and Phase 2 drug metabolism enzymes. These enzymes add functional groups to make lipophilic molecules more hydrophilic in order to make them easier to eliminate. The oxidative reaction is mainly catalysed by the Phase 1 cytochrome P450 (CYP) enzymes and the rest by the Phase 2 conjugating enzymes.

The CYP enzymes that play vital roles in the removal of xenobiotic pharmaceutical drugs from the human body belong to CYP 1, 2 and 3 sub-families. CYP metabolizing drugs are CYP 1A2, 2A6, 2C9, 2C19, 2D6, 2E1, and 3A4. 70% of these CYPs are localized in the human liver where 30% is constituted by CYP3A4 and 20% by CYP2C sub-family of enzymes.

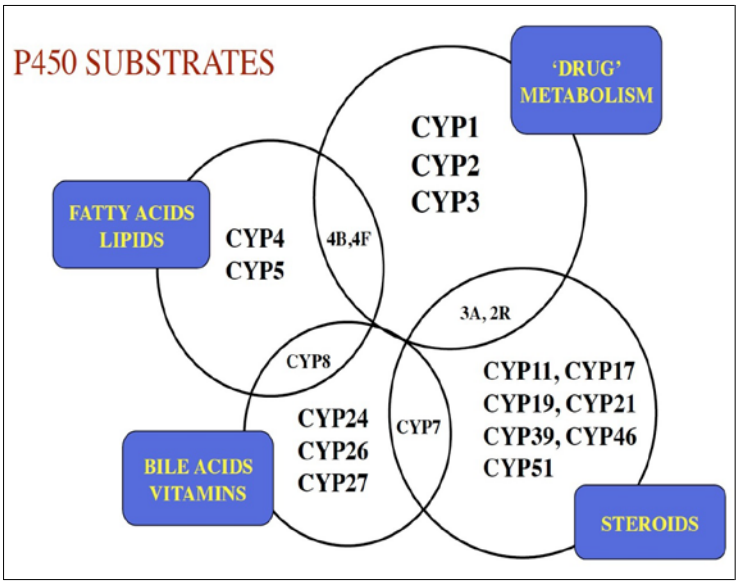


Figure 1.3. The CYP subfamily enzymes play different roles by acting on diverse sets of substrates. Besides being used for drug metabolism studies, the CYP 1, 2 and 3 sub-families of enzymes have other roles in the human body. Members of certain sub-families have been shown to have overlapping functions in being able to act on two different sets of substrates (MEDCH, 2013).

CYPs play important roles in endogenous biochemical pathways such as steroid hormone biosynthesis, metabolism of polyunsaturated fatty acids (such as arachidonic acid and prostaglandins) and activation of vitamins A and D_3 to biologically active hormones. They also participate in the activation and detoxification of a variety of different types of exogenous chemicals, some of which possess medicinal properties and others which are known to contaminate or pollute the environment (Figure 1.3; Estabrook 2003).

Mutations in *CYP* genes that correlate with deficiencies in enzyme activities are responsible for several human diseases (Murata 2001). CYPs can also show great inter-individual variability mainly due to environmental factors (e.g. CYP2E1 being induced in individuals who drink ethanol and CYP1A1/CYP1A2/CYP1B1 being induced in individuals who are exposed to aromatic hydrocarbons), genetic factors (that could result in specific CYP enzyme deficiencies) and patho-physiological factors (CYP2E1 being induced in diabetics).

Induction of CYP1 family of genes (which is induced by polycyclic hydrocarbons found in cigarette smoke and charred food) is a risk factor in several cancers such as lung and colon cancers because these enzymes can convert pro-carcinogens to carcinogens. Mutations in the *CYP1B1* gene (leading to defects in CYP1B1 activity) are linked to congenital glaucoma possibly because of the enzyme's major role in steroidogenesis. A metabolite formed from acetaminophen (Paracetamol) by CYP2E1 can cause severe toxic effects in humans. People who are alcoholic are at high risk to Paracetamol toxicity because they possess alcohol-induced high levels of CYP2E1. CYP2C8 is selectively responsible for inactivation of the anti-cancer drug paclitaxel (Taxol) through oxidation to 6α-hydroxypaclitaxel. Specific alleles of CYP2C8 are known to have a great influence on the pharmacokinetics of paclitaxel (Nakajima et al. 2005). CYP2C19 is known to metabolise a proton pump inhibitor, omeprazole, which is used to treat peptic ulcer. Omeprazole has been used as a CYP2C19 marker to assess gene-dosage effects and its intra-subject variability (Yin et al. 2004).

Some drug molecules can activate or inhibit the CYP enzymes. This can lead to adverse effects of drugs on the human body. For example if ketoconazole (an antifungal

compound), a CYP3A4 inhibitor, is administered together with Triazolam (sedative to treat insomnia) which is metabolised by CYP3A4, it will result in an overdose of Triazolam. This is an example of drug-drug interaction which often can cause major problems in the clinic. New drug candidates with poor metabolism profile or which cause drug-drug interactions often do not survive the drug development process which involves a lot of expense often leading to expenditures of $1 billion or more. Therefore, choosing drugs that do not interact with CYP enzymes is very important at an early stage of drug development. In recent years, the area of drug metabolism has become crucial to the drug discovery process. Use of drug metabolising enzymes early in the drug discovery process can save significant amount of costs and time required in pre-clinical and clinical studies, thereby greatly facilitating the process. That is the reason why introduction of drug metabolism screens as early as possible in the drug discovery process is gaining appeal. Drug metabolism studies have become as important as primary screening of a library of compounds against a therapeutic target, in the drug discovery process.

It appears that certain CYP enzymes have a high affinity towards chemical structures commonly found in pharmaceutical drugs. CYP2D6 and its genetic variants are known to metabolise a quarter of all known pharmaceutical entities (Werck and Feyereisen 2000). This subset of CYP isozymes is also considered to be highly important in drug metabolism studies because of their presence in the human liver, – the main organ involved in metabolism (see Table 1.3).

CYP3A4 is the most abundant CYP at ~30% of the total CYPs present in the human liver, the others existing in lower yet substantial amounts (1-15%) (Gonzalez 1992; Beaune 1993).

Table 1.2. Pharmaceuticals, as substrates for different CYPs.

CYP	Xenobiotics Metabolised	Xenobiotics Activated	Inducers	Inhibitors
CYP1A2	Caffeine; 7-ethoxy-resorufin; 7-methoxy- resorufin; imipramine; phenacetin; theophylline; tacrine R-warfarin	Acetaminophen (paracetamol); aflatoxin B1; 2-aminoacetyl fluorine;	Omeprazole; cruciferous vegetables; cigarette smoking	Enoxacin; fluvoxamine; isosafrole; apigenin
CYP2C9	Chloramphenicol; diclofenac; ibuprofen; flurbiprofen; hexabarbital; naproxen; omeprazole; phenytoin; retinoids		Rifampin	Warfarin; tolbutamide; sulfaphenazoe
CYP2C19	Diazepam; hexobarbital; mephobarbital; omeprazole; propanolol; phenytoin		Rifampin	Omeprazole; mephenytoin
CYP2D6	Bufarolol; dextromethorp-han; desipramine; ethylmorphine; imipramine; metoprolol;			Quinidine; quinine; paroxetine; fluoxetine; norfluoxetine
CYP2E1	Acetaminophen; acrylonitrile; dapsone; enflurane; ethanol; halothane; isoflurane; p-nitrophenol	Acetamino-phenacrylonitrile; benzene; chloroform; nitrosamine; styrene; vinylbromide	Alcohol; acetone; pyrazole; isoniazid; obesity	Disulfiram; diethyldithio-carbamate; 4-methyl-pyrazole
CYP3A4	Alfentanil; antipyrine; chloropromazine;clarithromycin; cyclosporine-A; dapsone; dexamethasone; erythromycin	Acetaminophen aflatoxin B1;	Phenobarbital; dexa-methasone; rifampin; phenytoin; carbamazepie	Triacetyl-oleandomycin; clarithromycinerythromycin; gestodene; ketoconazole; clotrimazole; naringenin

1.8 CYP19A1 (Aromatase) – Its Role in Breast Cancer

Breast cancer is the most common type of cancer in women in Western countries. Estrogens (i.e. estradiol and estrone which is a derivative of estradiol) are key factors in the pathogenesis and progression of breast cancer. The key enzyme aromatase is required for the conversion of

- Androstendione to estrone, and
- Testosterone to estradiol (Figure 1.4).

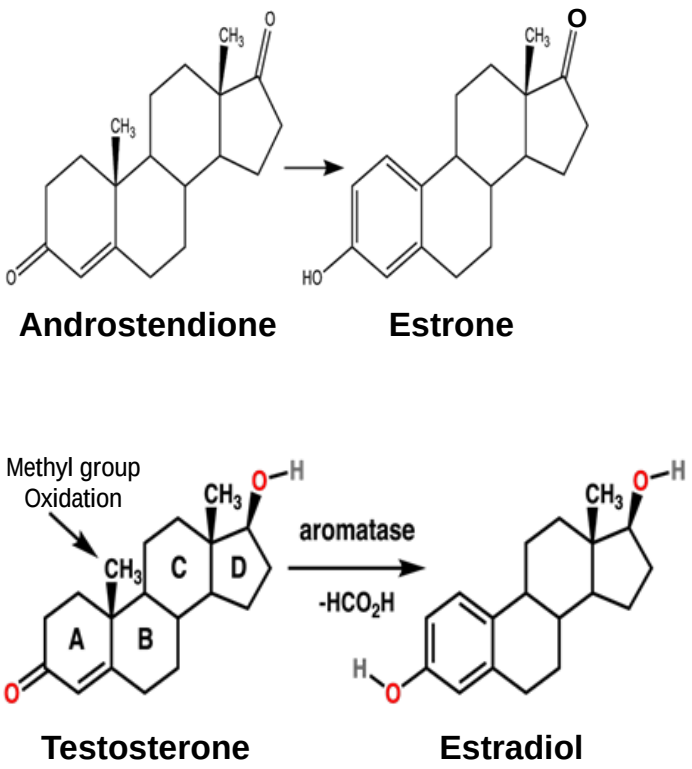


Figure 1.4. The role of aromatase in the syntheses of the steroids, estrone and estradiol.

Aromatase converts the ‘A’ ring of the steroids, androstendione and testosterone, to the aromatic state. These reactions take place primarily in ovarian granulosa cells but also in many other tissues (S.E. Bulun *et al*., 2005). The aromatase enzyme is coded by the cytochrome P450- gene, *CYP19A1*. The CYP19A1 protein, like most other CYPs, is anchored to the endoplasmic reticular membranes of estrogen producing cells. Again, like other human CYPs it is activated by NADPH-dependent cytochrome P450 reductase (CPR).

Aromatase inhibitors block the peripheral conversion of androgens (androstendione and testosterone) to estrogen (estrone and estradiol) and thereby reduce estrogen levels in tissue and plasma. There are two major types of aromatase inhibitors, type 1 steroidal (i.e. exemestane) drugs which bind competitively and irreversibly to the enzyme and are inactivators. The other type consists of non-steroidal inhibitors (i.e. anastrazole) and bind reversibly.

The main primary source of estrogen in the postmenopausal women is the conversion of circulating androgens via aromatization at peripheral sites (i.e. adipose tissue, skin) (Montagna *et al*., 2013). One of the focuses of this study was to create DNA constructs that would allow overexpression of CYP19A1 and its isolation as a microsomal enzyme. The aim was, with the ready availability of CYP19A1 enzyme in abundance, new non-toxic inhibitors could be identified from natural product libraries derived perhaps from edible plants. Aromatase inhibition is currently the most effective treatment of postmenopausal breast cancer, as they reduce aromatase activity throughout the body, resulting in breast cells also being deprived of estrogens.

Treatment of breast cancer with the currently available aromatase inhibitors, over a long period of time, often leads to resistance due to over-activation of specific kinases related to cell signalling, MEK, RAF, P13K, mTOR (mammalian target of rapamycin), and Akt (Chumsri *et al*., 2011). Hence, second generation inhibitors are widely sought and are of great interest.

1.9 CYP17A1 (17α-hydroxylase/17, 20 desmolase) – Its Role in Prostate Cancer

Another aspect of this study was to produce the human CYP17A1 enzyme in baker's yeast. CYP17A1 is over-expressed in prostate cancer and plays a major role in its development. Inhibition of this enzyme which activates genes, required for normal male sexual function, can prevent prostate cancer development in men.

Under normal conditions, dihydrotestosterone [DHT; also known as androstanolone (5α-androstan-17β-ol-3-one] is synthesized predominantly by 5α-reduction of testosterone.

This depends on the affinity binding of DHT (an androgen) to the androgen receptor (AR), which is a transcription factor that controls androgen-dependant gene transcription.

CYP17A1 enzyme has dual function in the intracrine synthesis of testosterone and DHT from progesterone via the cholesterol pathway (see Figure 1.5).

Its 17α-hydroxylase function acts on pregnenolone, a progesterone precursor, by adding a –OH group on carbon 17 of the steroid D ring. Its 17,20-lyase activity acts on 17-hydroxyprogesterone and 17-hydroxypregnenolone to split the side chain of the steroid

nucleus and produce DHT via 5α-pregnane-3α,17α-diol-20-one, through a backdoor pathway.

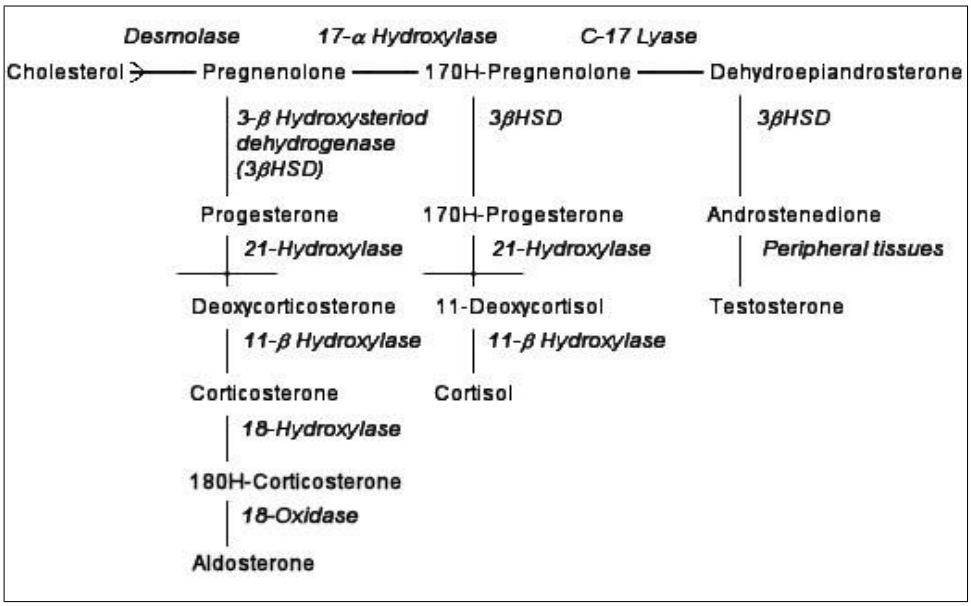


Figure 1.5. The pathways that lead to the synthesis of androgens that act as ligands for the androgen receptor which is mis-regulated in prostate cancer (Mula-Abed *et al,* 2013).

Recently, abiraterone has been approved for the treatment of prostate cancer. It selectively inhibits the CYP17A1 enzyme that is vital for androgen synthesis. Several studies have shown that abiraterone blocks the growth of prostate cancer cells (Siegal at al., 2012). The forces behind the progression to metastatic castration-resistant prostate cancer (CRPC) are multi-factorial but the main contribution is the persistence of androgen from non-gonadal sources that leads to intra-tumoral AR (androgen receptor) signalling (De Bono et al., 2011).

1.10 Expression of Human CYPs in Baker's Yeast

Cytochrome P450s (CYPs) are present in all mammalian tissues and is involved in the synthesis of endogenous compounds such as steroids, fatty acid and prostaglandins. They also participate in activating and detoxification of foreign compounds, namely chemical pro-carcinogens, environmental pollutants and many therapeutic drugs. CYP enzymes partake in the oxido-reductive catalysis cycle on the endoplasmic reticular membranes. As an electron donor, it has been suggested that the cytochrome P450 reductase (CPR) is surrounded by a circle of multiple CYPs. Using NADPH as a substrate, the CPR contributes once to the transfer of one electron to a particular CYP, and then unlinks itself from the first CYP to contribute an electron to the next one (Backes and Eyer, 1989; Eyer and Backes, 1992; Taniguchi and Pyerin, 1988; Taniguchi et al., 1979).

When a mammalian CYP is expressed in heterologous cells (i.e. in an organism different from the one where it naturally occurs), its activity is affected greatly by the amount of CPR present in the recombinant expression system. It has been reported that CPR is the key rate limiting factor for the catalytic activities of CYPs while phospholipids act as matrices for the interaction of CYPs and CPR that allow modulation of CYP activities (CYPs = enzymes, CPR = electron donor, and phospholipids = provide the membrane structure for binding of a CYP and the CPR).

Among heterologous expression systems, *Saccharomyces cerevisiae* was the first yeast that was used and applied further for the study of drug metabolism of human CYPs *in vitro* (Cheng *et al*., 2006). Although other organisms (such as *E. coli*, mammalian and insect cells) have been used for human CYP expression, yeast has specific advantages over the other expression systems:

- Ease of growth of yeast cells,
- Low endogenous CYP activity level,
- Intracellular membrane structure similar that bear phospholipids, and
- Endogenous CPR activity which can couple with human CYPs to provide CYP activity (Andrew. 2002).

Regardless of the advantages in yeast, one major problem of using yeast as well as other expression systems is the insufficiency of CPR activity in host cells. The endogenous level of the yeast CPR does not exceed a molar ratio of 0.05-0.1 relative to heterologous CYPs, and the in vitro activity is poor. Co-expression of CYPs with CPR, driven by strong yeast promoters, was reported as an approach to improve CYP activities. Besides, the co-expression of cytochrome b5 during expression in yeast has helped to stimulate the activities of certain CYPs towards some substrates (Murataliev *et al*., 2004). The stability of the heterologous expression system is a key factor for obtaining accurate in vitro data from drug metabolism studies.

1.10.1 CYP expression from episomal, extra-chromosomal vectors versus from chromosomally-integrated genetic copies

CYP protein levels may vacillate when human *CYP* genes are expressed from episomal vectors (such as pYEP plasmids) from yeast cells which contain the *CPR* gene expression cassette on a chromosomal locus. The vacillation depends on how toxic the human CYP protein is for the host organism in which it is being expressed. This is because the autonomously replicating sequence based on the yeast 2µ-plasmid allows for fluctuation of the *CYP* gene copy number depending on the cells' ability to cope with expression of a relatively toxic protein. Moreover, episomal plasmids are maintained within the cell during cell division through selection pressure using selective minimal media for growth of yeast cells.

In contrast, expression of heterologous CYP proteins from genomic copies of *CYP* genes, integrated into the yeast genome via homologous recombination, is likely to be far 'more stable' than expression from episomal vectors because

- There would not be any copy number fluctuations when *CYP* gene expression cassette(s) are integrated into the yeast genome (the copy number will solely depend on how many copies of the *CYP* gene expression cassette have been introduced into different chromosomal loci), and

- There would not be any necessity of growing cells in selective minimal media once the *CYP* gene expression cassette has stably integrated into one or more of yeast's chromosomes, hence allowing cells to be grown in a full nutritious

medium without any selection.

This would permit use of such chromosomally integrated expression systems for

- High-throughput screening of potential CYP inhibitors using whole cells, and
- Production of CYP proteins on a large scale.

As a proof of concept,

- Integrative plasmids for integration of the human *CYP3A4* gene (synthesized using yeast biased codons) were constructed and integrated into the yeast genome at different chromosomal loci, and then
- The CYP activities of yeast transformants, obtained from *CYP3A4* gene integrations, were compared with transformants that expressed the same gene from an episomal plasmid.

Both integrative and episomal expression systems used a modified, non-toxic (patented) version of human CPR for co-expression with CYP. Both systems used the inducible *ADH2* promoter (derived from one of the two alcohol dehydrogenase genes present in yeast) for expression of heterologous proteins.

1.10.2 Homologous recombination

This is a type of genetic recombination where genetic sequences are exchanged between two or more similar or identical molecules of DNA. The traditional use of baker's yeast *S. cerevisiae* in alcoholic fermentation has over time resulted in enormous accumulated

knowledge concerning genetics, physiology and biochemistry as well as genetic engineering and fermentation technologies. Yeast is widely being used in the sysntheses of new metabolic products through the development of new yeast strains (Nevoigt, 2008). The emergence of synthetic biology has introduced new ideas on how foreign DNA can be inserted into the yeast genome. Incorporating complete natural or synthetic pathways to the production of a chemical into yeast has been attained but still remains a major challenge that is currently hindering the synthesis of any new therapeutic molecule of choice. The optimization of a newly developed technology is always hard to achieve. Different combinatorial methods for inserting foreign genes into yeast's genome have been deployed. The major advantage in using homologous recombination comes from the observation that proteins can be stably expressed with correct folds independent of the proteins' primary sequence which can be sometimes a problem during expression from an extra-chromosomal plasmid (Martina et al., 2007)

1.11 CYP1 Family of Enzymes

Three genes are members of the CYP1 family: *CYP1A1*, *CYP1B1* and *CYP1A2*. According to Nelson, (1996), this family has no pseudogenes and they all share the same features of regulation; all are transcriptionally controlled by the AhR-ARNT transcription factor complex (Scmidt, 1996). AhR (aryl hydrocarbon receptor) is a hormone receptor which acts as a transcription factor upon binding to its ligand. ARNT (aryl hydrocarbon receptor nuclear translocator) is a co-activator of AhR. Induction of gene transcription by the AhR-ARNT complex occurs through binding of polycyclic aromatic hydrocarbons (PAHs) to AhR which act as ligands to the transcription factor

complex. The AhR-ARNT transcription activator complex activates genes of the CYP1 family (Figure 1.6).

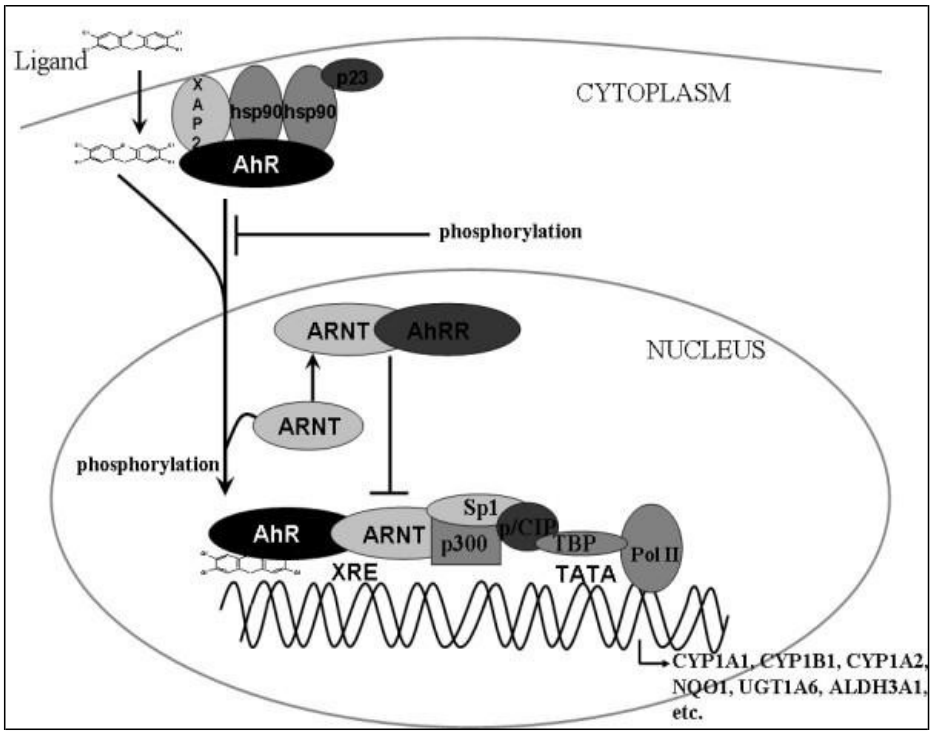


Figure 1.6. Transcription activation of CYP1A1, CYP1B1 and CYP1A2 genes through induction by the AhR-ARNT transcription activator complex, bound to a PAH (a polyaromatic hydrocarbon), (Vasilis P et al., 2009).

CYP1A1 activates mainly two different types of pro-carcinogens, PAHs and heterocyclic amines, to carcinogens.

CYP1A1's mechanism of activation of the pro-carcinogen benzo[a]pyrene (a PAH) is through its oxidation to benzo[a]pyrene 7,8-oxide [β(a)P-7,8-oxide], and subsequent hydrolysis to the two enantiomeric diols (+)-β(a)P-7,8-diol and (-)-β(a)P-7,8-diol (Vasilis P et al., 2009).

CYP1A1's mechanism of activation of the pro-carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is depicted in Figure 1.7.

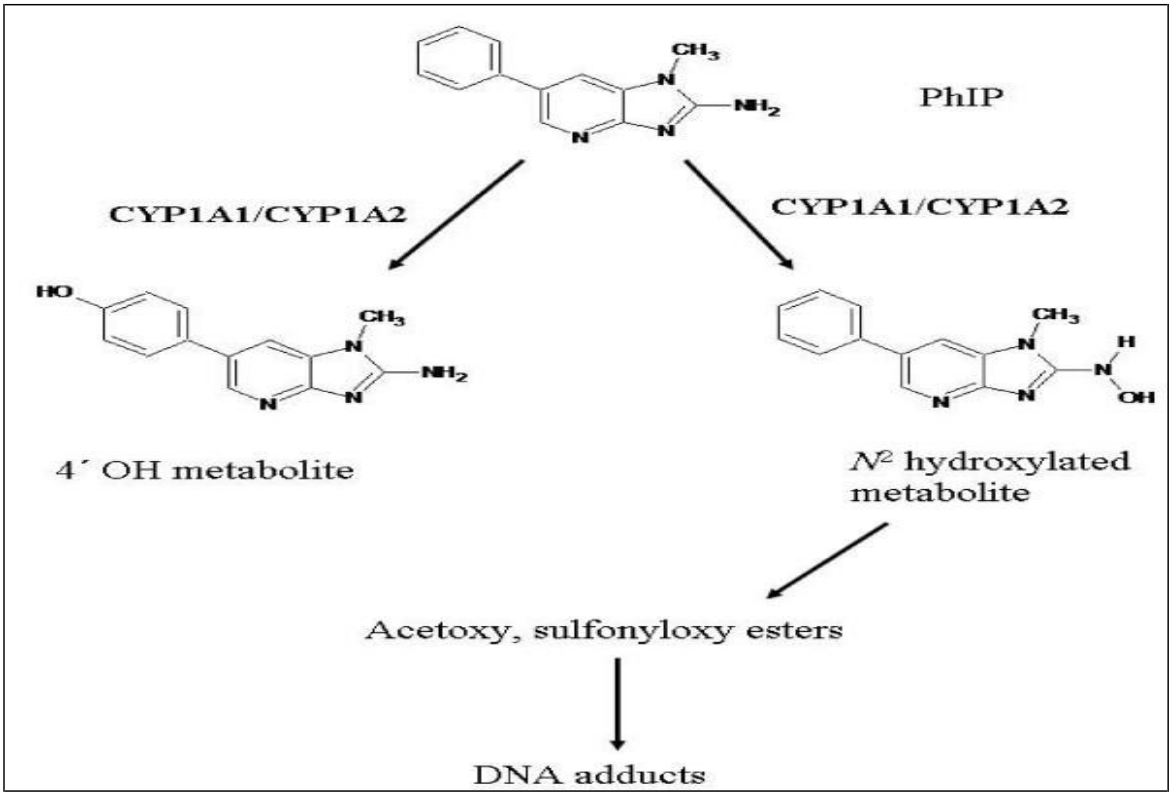


Figure 1.7. Activation of the potential *pro-carcinogen* 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (*PhIP*) to the N-hydroxylated metabolite which is a carcinogen (Vasilis P et al., 2009).

The expression of CYP1A1 in the human liver is very low (Edward, 1998) compared to the expression of CYP1A2 which is liver specific. The CYP1A2 protein has not been detected in any other tissue besides the liver. CYP1A2 constitutes about 13% of the total hepatic CYP content (Imoka, 1996).

Similar to CYP1A1, CYP1B1 occurs at very low levels in the liver, kidney, prostate, mammary gland and the ovary (Murray, 1997). Like CYP1A1, CYP1B1 is also regulated by several key transcription factors, such as AhR-ARNT complex, the SpI transcription

factor, a cyclic AMP (Camp)-response element-binding protein (CREB), and possibly the estrogen receptor (Chang et al., 1998; Chen et al., 1996; Zheng et al., 2003). Once CYP1B1 is up-regulated, it catalyses the conversion of steroid hormones and exogenous substrates into toxic metabolites that increase the genotoxic and oxidative load on the cell and modulate cell signalling (Sissung *et al*., 2006). Hence, CYP1B1 has been thought to be a target for the discovery of new anticancer agents.

1.12 CYP2 Family of Enzymes

The human CYP2 proteins are a heterogeneous group of enzymes and do not seem to be regulated at the transcriptional level. The substrates and tissue specificity of the CYPs are also different from other CYP families. This study will focus on the CYP2C9, CYP2C18, CYP2C19, CYP2B6, CYP2J2 and CYP2D6 isozymes (together with the three SNPs of CYP2D6). I have successfully cloned genes coding for all these *CYP* genes in expression vectors that would allow production of these proteins in the yeast *Saccharomyces cerevisiae*.

I have shown that CYP2C18 does not require cytochrome b5 but its close homologue CYP2C19 requires b5 for superior activities.

The CYP2C subfamily contains four highly homologous proteins, 2C8, 2C9, 2C18, and 2C19, which are located in a cluster on chromosome 10 and this subfamily accounts for 20% of the human total liver CYP content (Imoka, 1996).

CYP2D6, a xenobiotic metabolizing cytochrome P450, less significantly present in the liver (only about 2% of the human hepatic CYP enzymes) but is significantly found on

the surface of the cell plasma membrane (Jacqueline et al., 1998). In the present study, I have explored the role of yeast-produced CYP2D6 as a drug metabolising enzyme using whole yeast cells for the biotransformation of codeine in the production of its metabolite, morphine. Although the amount of CYP2D6 in the liver is low, it is still involved in about 25% of the metabolism of drugs currently in use (Martina et al. 2012) . A suitable expression system has been devised in this study to express wild-type CYP2D6 and five other variants in whole cells. Microsomal CYP2D6 enzymes have been isolated from two of these variants, the wild-type (Met^{374}) and the Val^{374} counterpart. It has been reported that CYP2D6 does not require the co-expression of cytochrome b5 for its enzymatic activity. Here, I present evidence that if CYP2D6 is co-expressed with another enzyme (e.g. CYP3A4 in the presence of cytochrome b5), the expression of both these enzymes (CYP2D6 and CYP3A4) is much higher than when CYP2D6 is expressed on its own. An explanation to support this observation is provided in Chapter 7.

Another important family of CYP2 family member, CYP2B6, is present in the human liver. It is involved in the biotransformation of drugs (e.g. anticancer drugs such as cyclophosphamide) and other xenobiotics. In the past, CYP2B6 was reported to be present only in part of the general population but recent studies have shown that it is present in all adult human livers (Hoffmann et al., 2008). CYP2B6 is about 1-10% of the total hepatic CYP protein in individuals (Zanger et al., 2007). In Chapter 3 of this study, I have expressed human CYP2B6 enzyme in baker's yeast, using yeast biased codons, at high levels.

1.13 CYP3A Subfamily of Enzymes

The human subfamily CYP3A plays a dominant part in the metabolic elimination of pharmaceutical drugs. CYP3A enzymes are localized in the liver and the small intestine. Family members contribute to first-pass and systemic metabolism of drugs. The CYP3A subfamily consists of the proteins: CYP3A4, CYP3A5, CYP3A7 and CYP3A43.

Although there is no systematic analysis of the true extent of its contribution, it is generally accepted that the CYP3A4 enzyme plays the most important role in the metabolic elimination of more drugs than any other CYP isozyme. CYP3A4's major role in drug metabolism maybe due to its large active site that permits the binding of structurally diverse molecules (i.e. different molecules derived from different scaffolds). Among adults, CYP3A4 is the dominant CYP3A enzyme in the liver and other organs.

CYP3A5 and CYP3A7 are polymorphically expressed in the foetal liver where CYP3A4 is totally absent. Because both CYP3A4 and CYP3A5 share substrate specificity (e.g. midazolam), it is difficult to segregate the relative contribution of the two enzymes in CYP3A mediated metabolism. The catalytic activities of both CYP3A4 and CYP3A5 are affected significantly by the presence of cytochrome b5. The level of CYP3A4 activity expressed in a yeast expression system, with the yeast strain containing the human *cytochrome b5* gene has shown a 40-fold higher level of activity compared to the one expressed without cytochrome b5 (Zanger *et al*., 2013).

Expression of CYP3A4, and its stability and inhibition are regulated by different mechanisms (Figure 1.8). Many factors which include nuclear receptors, hormones,

xenobiotics and diverse signalling molecules regulate CYP3A4 expression (Zanger et al., 2013).

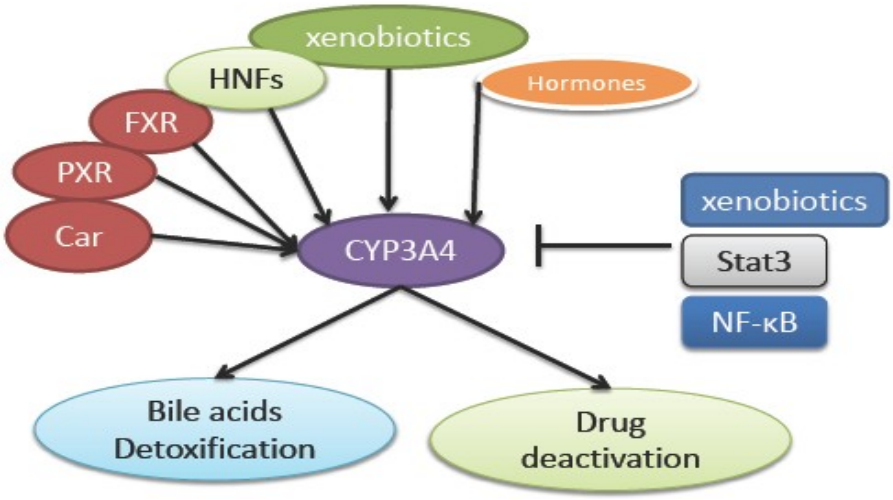


Figure 1.8. Pathways through which CYP3A4 are activated/deactivated, and the enzyme's role in deactivation and detoxification of substrates (Chen et al., 2014).

1.14 CPY4 Subfamily of Enzymes

CYP4 subfamily of enzymes is one of the oldest CYP450 sub-families which are thought to have been evolved about 1.25 billion years ago. It comprises of about 22 isoenzymes encoded by genes that are expressed either in a constitutive or an inducible manner. CYP4 enzymes are related to two functional aspects: (a) as cholesterol metabolizing enzymes and (b) as membrane integrity maintaining enzymes (Nebert and Gonzalez, 1985). I have constructed expression cassettes for the three CYP4 sub-family genes, *CYP4A11*, *CYP4F3* and *CYP4F2* genes, for expression in yeast. In this study, I have shown the cloning and expression of only the *CYP4F3A* gene.

1.14.1 The 4A Subfamily

The CYP4A set of isozymes is a sub-family of CYP4. Thirteen *CYP4A* genes have been identified in humans (Kawashima et al, 1992). They code for proteins that have the capability of hydroxylating terminal ω-carbon atoms of both saturated and unsaturated fatty acids (Figure 1.9). The CYP4A proteins also participate in prostaglandin synthesis (Yemamato et al., 1984). When the first member of the CYP4 gene family was isolated, cloned and sequenced (Hardwick et al., 1987), its amino acid sequence revealed that it was far less homologous (< 36%) to the CYP family of proteins known at that time. These enzymes are expressed constitutively in the liver, kidney and their expression is induced by clofibrate, a hypolipidemic, which lowers plasma triglyceride together with cholesterol levels.

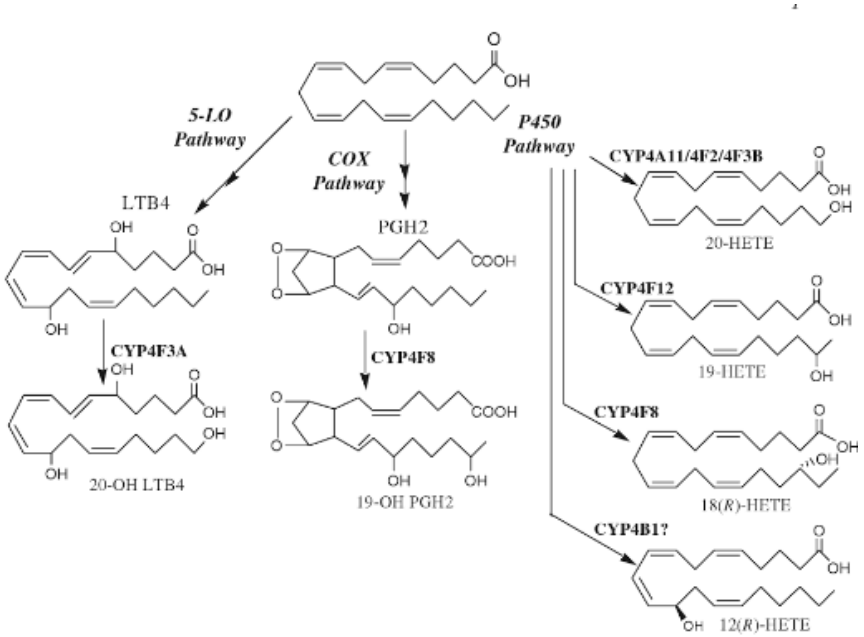


Figure 1.9. Human CYP4 enzymes that are involved in the metabolism of arachidonic acid. The diagram shows the CYP4 enzymes that metabolise eicosanoid products within the 5-lipoxygenase, cyclooxygenase and CYP450 pathways.

It has been reported that CYP4A-mediated conversion to metabolites often occurs with high degree of regioselectivity (Kroetz et al ,. 2005; Cosima et al 2010). For example, in humans, CYP4A11 uniquely hydroxylates lauric acid. It seems that CYP4A enzymes generally require cytochrome b5 for maximal turnover. It has been reported that CYP4A11 has a role in the formation of 20-hydroxyeicosatetraenoic acid (20-HETE) as a metabolite in the human liver and kidney (Cosima et al 2010), HETE serves as a second messenger different hormones and growth factors and plays partial opposing roles in the regulation of vascular, renal and cardiac function (Fleming , 2008). CYP4A11, which is also referred to as 20-HETE synthase, is considered to be a major contributor to the formation of 20-HETE in the human liver rather than the kidney. In contrast, CYP4F2 may be the dominant 20-HETE synthase in both liver and kidney.

1.14.2 CYP4F Subfamily

The genes of the human CYPF subfamily are mostly located on chromosome 19p13.1-2 (Kikutal *et al*., 2002). The *CYP4FA11* gene is the exception, being located on chromosome 1 (Nelson et al., 1996). The major genomic difference between *CYPF3* and *CYPF4* genes is that the pre-mRNAs of the former set of genes alternatively use exons 3 or 4 which lead to the formation of the 2 variants CYP4F3A and CYP4F3B.

In this study, I have elaborated on the construction of the plasmids that express the CYP4F3A enzyme. The *CYP4F3A* mRNA uses exon 4. The CYP4F3A enzyme metabolises mainly leukotriene B4 rather than arachidonic acid but the variant CYP4F3B

which preferentially uses exon 3 shifts the metabolism towards synthesis of arachidonic acid (Christmas *et al*., 1999).

Mutations in the human *CYP4F2* gene have been linked to elevated blood pressure. This enzyme also contributes to the biosynthesis of 20-HETE. The natriuretic and anti-hypertensive effects of the unsaturated fatty acid, 20-HETE, fail to be manifested in humans carrying this genetic disorder. *CYP4F2* gene mutations have been reported to induce a change in warfin doses (Matthew et al., 2009). Cardwell et al., (2008) have suggested that the reaction was mediated indirectly through 20-HETE production, a reaction catalysed by CYP4F2.

Vitamin K1 has been shown to be a substrate of the CYP4F2 enzyme. It was found to catalyse formation of a new metabolite of vitamin K1 when screening against other recombinant CYP450 enzymes (Figure 1.10; Rettie and Tai, 2006, Sontag and Parker 2002, 2007; Hardwick, 2008).

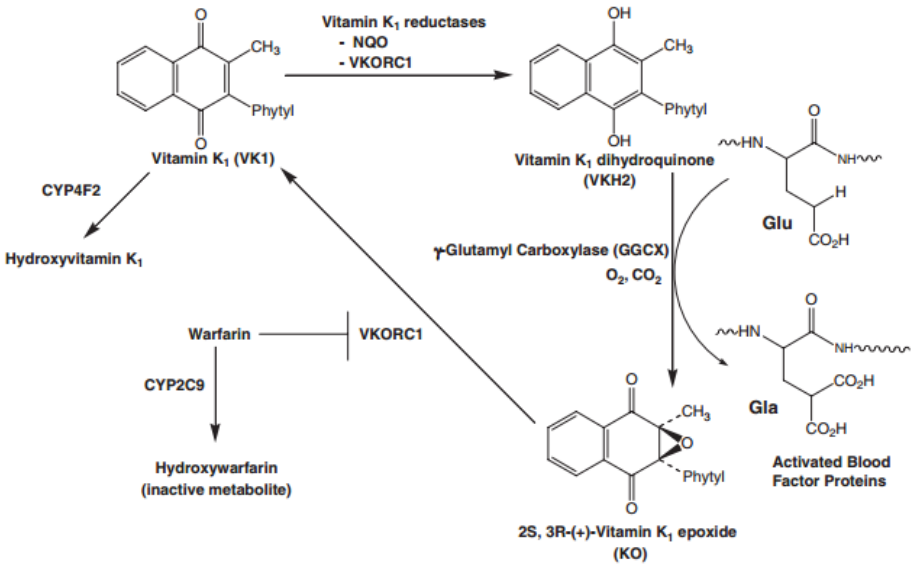


Figure 1.10. Role of CYP4F2 in vitamin K1 homeostasis. Vitamin K1 dihydroquinone, a cofactor of glutamyl carbonxylase, is responsible for activation of several blood factor proteins through carboxylation of key glutamic (Glu; E) amino acid residues that initiate the blood coagulation cascade response. Both CYP2C9 and CYP4F2 enzymes affect warfarin dose responses by controlling clearance of warfarin and removal of vitamin K1 from the system (Rettie and Tai, 2006).

1.15 Microsome preparation from human liver tissues

Many early studies have utilized CYP450 enzymes purified from the human liver (Distlerath 1985; Yun 1991). Human liver microsomes are available, although not very widely, from a few commercial companies that sell these preparations. If properly handled, liver tissues obtained after surgery or from organ donors can be frozen in liquid nitrogen and held at -80ºC essentially indefinitely without apparent loss of P450 enzyme activity (Pearce 1996). The microsomal preparations can also be stored for long periods. But the availability of tissues and the technical difficulties in purifying individual or total enzyme activities are a big problem. Moreover, the total CYP activity in isolated livers

seems to disappear very quickly. These problems have caused a shift to heterologous methods that allow production of CYPs in specific cell systems for diverse practical applications in drug metabolism studies (Guengerich 1991; Miners 1994).

Catalytically active cytochrome P450 enzymes have been successfully expressed in bacteria (*Escherichia coli*), insect cells, yeast (*Saccharomyces cerevisiae,* and to a lesser extent *Schizosaccharomyces pombe*) and mammalian cells. These heterologous systems can be used to express human CYP enzymes along or conjunction with one or both of their redox partners. All expression systems have their unique advantages and disadvantages. The following section will discuss and compare the different heterologous recombinant systems.

1.16 Recombinant P450 expression systems

1.16.1 Expression systems based on mammalian cells

In theory, a mammalian cell expression system should be the ideal system to express CYP enzymes. In mammalian cells, only the catalytic part needs to be produced. This is the part which disappears (or is non-existent) in most mammalian cells in culture (Crespi 1993). The P450 reductase (CPR) remains functional in mammalian cells but in tiny amounts. Therefore, extra amounts usually need to be produced for CYP overexpression in mammalian cells. Unfortunately, by the nature of its biochemical activity (i.e. ability to generate free electrons), CPR is toxic for cells.

Stable heterologous expression of individual human CYP450s have been achieved in several cell lines, including V79 Chinese hamster (Doehmer 1991), Chinese hamster ovary (CHO) cells (Ding 2001), HepG2 cells (Aoyama 1990), NIH3T3 cells (Battula 1987), and human lymphoblastoid cells (Crespi 1991). At some yet undefined level (which is very small), most CYP enzymes appear to adversely affect the growth of host mammalian cells (Crespi 1993). Because of their effect on growth, these low levels would represent the upper limit of expression that can be achieved in mammalian cells. Within the bounds of this limitation, there is the possibility to exploit the presence of native levels of CPR and cytochrome b_5/ cytochrome b_5 reductase for the expression of CYP enzymes, without the need for co-expression of the co-factor proteins. However, mammalian systems that constitutively express CYPs are quite unstable for reasons which are not clear. Instability implies that either the genetic information is quickly discarded by the cells (i.e. it is lost) or cells fail to express any recognizable level of CYP enzymes.

However, a distinct advantage of the mammalian cell expression system is that it offers the possibility to couple CYP metabolite formation to toxicological endpoints that is, cytotoxicity, malignant transformation, or mutagenesis investigations (Sakaki 2000). It should be noted that similar toxicological endpoints could also be measured in yeast based systems if such systems were to be available.

The disadvantages of a mammalian cell expression system are that expression levels are low, work in mammalian cell culture is extremely expensive and sensitive assays are needed. For reasons that are also poorly understood, most human CYP cDNAs do not yield any significant level of expressed protein in the mammalian cell expression systems that have been reported until now.

1.16.2 Bacterial cell expression systems

Bacteria (*Escherichia coli*) are the most commonly used host organism for expression of cytochrome P450 enzymes. There are many advantages for using a bacterial expression system. Firstly, development of a bacterial system is rapid and the culture medium is inexpensive relative to mammalian or insect cell systems. Secondly, the bacterial system is ideal for the isolation of large quantities of enzyme for spectroscopic or structural studies. Thirdly, purification of bacterially-expressed CYPs is relatively straightforward. The purified CYPs can be used with CPR and, when necessary, with cytochrome b_5 to reconstitute catalytic activities at expected rates.

CYPs made in bacteria have been used for a variety of purposes. For example, readily available large amounts of CYPs, purified from bacteria, have been used as antigens for the preparation of polyclonal antibodies. The oxidation products generated via a relatively short 20 min incubation of a substrate (bufuralol) with large amounts (35 nmol) of a recombinant CYP (i.e. CYP1A2) can rapidly be isolated and characterized by mass and ^{1}H-NMR spectroscopy (Yamazaki 1994; Dong 1996). However, the greatest disadvantage of the bacterial system is that bacteria lack the endoplasmic reticulum to which both mammalian microsomal CYPs and CPR (the electron transfer partner for microsomal CYP activity) bind to manifest their activities.

At least 35 mammalian CYPs (human and non-human) have now been expressed in *E. coli*. Bacterial expression uses two distinct approaches: either (a) the sequence of the proteins is modified at the N-terminus (through minor changes in the amino acid sequence

and often truncation of the 35-51 amino acid membrane anchor domain that lies at the N-terminus) so as to obtain soluble, unanchored proteins (Iwata 1998) or (b) *ompA* targeting sequences are incorporated at the N-terminus to direct CYPs and the CPR to the periplasmic membranes of bacteria, where the signal sequence is removed by proteolytic cleavage (Princhard 1998). With either approach, appropriate choice of the host strain is essential for efficient cDNA expression. Although it is believed that modifications at the N-terminal region of the CYP protein and the CPR are not important for substrate recognition or catalysis, extensive validation of kinetic properties of expressed protein is definitely prudent. In general, the reported rates of catalysis for isolated bacterial expressed CYPs are lower than those observed with proteins obtained from other systems.

In human cells, the two subunits, CYP and CPR, are always separate (i.e. they are not physically associated but act in "trans"). However, often fusion proteins between a particular CYP and the CPR have been produced in bacteria and some of them have been purified (Shet 1993; Parikh 1997; Iwata 1998). A fusion protein is a single-chain protein that contains both the P450 (CYP) and NADPH-P450 reductase (CPR) subunits covalently joined together. The catalytic activities of these available fusion proteins have not been extensively investigated. Initial investigations that have been reported indicate that the fusion proteins are quite unstable.

1.16.3 Expression systems based on yeast cells

The first system used to successfully express a mammalian P450 was yeast. Heterologous expression of mammalian P450 enzymes in yeast was first reported by Ohkawa and his colleagues in Japan (Sakaki 1985). In principle, yeast has the possibility of becoming one of the best cell systems for expressing or producing P450 enzymes. Yeast is the only heterologous system that contains very similar intracellular organelles to that present in human cells (Pompom 1997). Mammalian P450 proteins are found in subcellular membranes in human cells, such as mitochondria and endoplasmic reticulum and require electron transfer enzymes, NADPH-ferredoxin reductase for mitochondrial P450-s, and NADPH-P450 reductase for microsomal (i.e. endoplasmic reticular) P450 enzymes, to exhibit their catalytic activities. It is highly desirable that heterologous host cells contain human cell-like intracellular compartments and electron transfer enzymes in order for the human P450 enzymes to be produced with activities that are very similar to that present in human cells. In this respect, baker's yeast (Saccharomyces cerevisiae) is very similar in its intra-cellular architecture and electron transfer enzymes to that of human cells.

Yeast also has its own NADPH-P450 reductase (CPR) and cytochrome b5 indicating that yeast has the perfect machinery to produce active human CYPs and CPR. Therefore, one can envisage co-producing the catalytic partner (i.e. a CYP) along with one or both reductases (i.e. the yeast and the human *CPR* genes) in yeast. While the expression of human CYPs in *Schizosaccharomyces prombe* (Ehmer 2002), *Pichia pastoris* (Anderson 2002) and *Yarrowia lipolytica* (Nthangeni 2004) has been reported, the baker's yeast *Saccharomyces cerevisiae* (Ching 1991; Pompon 1996; Pompom 1997; Hayashi 2000)

has proved to be the yeast most often used for heterologous expression of human CYP proteins.

Lower cost of growing yeast compares very favourably with mammalian cells and insect cell based systems. Moreover, yeast can be grown to comparable densities to bacterial cells and much higher densities than insect cells. The observed P450 expression levels are high enough to allow both spectrophotometric and detailed biochemical analysis of single human CYPs directly in yeast microsomal fractions.

Based on the above, yeast is potentially a better host cell than bacteria and insect cells for expression of P450 enzymes. In the past, the activities of human CYP enzymes, expressed in yeast, were the major problem. In the present study, I have been able to use the concept of expressing human genes, synthesised with yeast biased codons, to avert this problem. The results have provided a good response with high CYP enzymes activities.

1.16.4 Baculovirus Expression System

First foreign gene expression with a baculovirus was demonstrated more than 20 years ago (Smith et al., 1983). Traditionally, recombinant baculovirus generation is carried out in two steps (Berger, 2004): (1) gene of interest is cloned into a small transfer vector and propagated in *E. coli* and, (2) later cloned into the baculovirus genome by homologous recombination in insect cells yielding 30-80 recombinant progenies (O'Reilly et al., 1994). The major drawback of the baculovirus system is that, once a composite baculovirus is constructed, it cannot be modified easily, partly due to its large size (130 kb). Moreover, proteins produced by the baculovirus system are not always amenable to

X-ray crystal studies. The proteins need to be truncated or mutated before they can be coaxed into forming highly order single crystals (Trowitzcsh wt al., 2010).

1.17 Introduction to the yeast expression system

1.17.1 Saccharomyces cerevisiae is a model eukaryote

Although yeasts have greater genetic complexity than bacteria, containing 3.5 times more DNA than *Escherichia coli* cells, they share many of the technical advantages that permitted rapid progress in the molecular genetics of prokaryotes and their viruses. Some of the properties that make yeast particularly suitable for biological studies include rapid growth, dispersed cells, the ease of replica plating and mutant isolation, a well-defined genetic system, and most importantly, a highly versatile DNA transformation system (Daum 2000; Buschini 2003). Unlike many other microorganisms, *S. cerevisiae* is viable even after introduction of numerous auxotrophic markers. Being non-pathogenic, baker's yeast (henceforth will be referred to simply as "yeast") can be handled with fewer precautions. Moreover, large quantities of yeast can be grown in fermentors and therefore yeast-produced heterologous proteins can be a cheap source for further biochemical studies on human proteins (Nishihara 1997; Masimirembwa 1999).

Unlike most other microorganisims, strains of *S. cerevisiae* have both a stable haploid and diploid state. Thus, recessive mutations can be conveniently isolated and manifested in haploid strains, and complementation tests can be carried out in diploid strains. The development of DNA transformation has made yeast particularly accessible to gene cloning and genetic engineering techniques. Structural genes corresponding to virtually

any genetic trait can be identified by complementation in yeast with wild type genes from plasmid libraries since the majority of the 6000 genes identified in yeast have homologues in human cells. Also, plasmids can be introduced into yeast either as extra-chromosomal replicating entities or by integration into the genome.

Integrative recombination of DNA through transformation (i.e. transfection) in yeast proceeds exclusively via homologous recombination. Techniques (i.e. protocols) similar to that developed for yeast have been used for genomic integration of genes in mammalian cells (Adams 1997). Via homologous recombination, exogenous DNA with segments homologous to that present in the organism's genome can be directed at will to specific locations in the host's genome. Homologous recombination, coupled with yeast's high levels of gene conversion, has also led to the development of techniques for the direct replacement of genetically engineered yeast DNA sequences into their normal chromosomal locations. Thus, normal wild type genes, even those having no previously known mutations, can be conveniently replaced with altered or disrupted alleles. The phenotypes arising after disruption of yeast genes have contributed significantly toward understanding the function of certain proteins in vivo.

Yeast transformation can be carried out directly with synthetic genes, permitting the convenient production of numerous altered forms of proteins. These techniques have been extensively exploited in the analysis of gene regulation, structure-function relationships of proteins, chromosome structure, and other general questions in cell biology. The overriding virtues of yeast are illustrated by the fact that mammalian genes can be introduced into yeast for systematic analyses of functions of the corresponding gene products.

In addition, yeast has proved to be invaluable for studies of proteins from other organisms which includes the use of the two-hybrid screening system for general detection of protein-protein interactions (Field 1989). Yeast artificial chromosomes (YACs) have been used for cloning large fragments of DNA. Yeast has also been used for elucidation of biochemical pathways in humans and other organisms. Most notably, these involve the cancer cell division cycle, elucidation of the function of protein kinases, proteolysis and protein degradation, protein folding/misfolding, apoptosis, chalking out the mechanism of action of a pharmaceutical drug, a toxic chemical or an agricultural herbicide (Bähler 2005). Besides the above, yeast expression systems have been used for lab and commercial scale preparations of heterologous proteins.

1.17.2 Yeast transformation

Saccharomyces cerevisiae is unique among eukaryotes in the ease with which it can be transformed with DNA, and the high frequency with which the introduced DNA can undergo homologous recombination with genomic DNA. There are several requirements for a successful transformation experiment (Adams 1997): (1) a means of introducing yeast DNA into cells; (2) a selectable marker on the introduced DNA with corresponding non-reverting mutations in the chromosomes that would allow generation of the auxotrophic markers; and (3) a vector system (i.e. a shuttle vector) that would allow propagation of cloned DNA in both *E. coli* and yeast.

1.17.3 Yeast selectable markers

Although the frequency of transformation of yeast can be quite high compared to other similar organisms, only a small fraction of the total number of cells in a transformation experiment are transformed. It is essential to have in yeast a selectable marker that would allow selection of cells that become transformed. All standard yeast vectors have a bacterial origin of replication and a bacterial selectable marker, usually the ß-lactamase gene that confers ampicillin resistance. At the same time, all standard yeast vectors also have a yeast selectable marker, such as a fully functional *LEU2*, *URA3*, *ADE2*, *HIS3*, *TRP1* or *LYS2* gene *etc*.(Sikorski 1989). In addition, all yeast strains contain markers that allow selection of transformants containing the desired plasmid. The most commonly used yeast markers consist of inactive (i.e. defective) *LEU2, URA3, ADE2, HIS3, TRP1* and *LYS2* genes which are usually represented as *ura3, his3, leu2, trp1* and *lys2* (the defective genes are always represented in lower casing). Mutations in these particular genes are chosen because of their low reversion rate. Plasmids containing functional *LEU2, URA3, ADE2, HIS3, TRP1* or *LYS2* gene can complement the corresponding inactive genes in the yeast strain. Moreover, these yeast markers can complement specific *E. coli* auxotrophic mutations and can thereby be used sometimes directly for selection in bacteria.

In this project, the basic yeast strain BC300 (derived from W303-1a; ATCC #208352) has been used extensively to generate BC300 derivatives. For example, the gene expression cassettes for a modified human reductase, ΔhRDM, have been introduced at the *LEU2* locus of BC300 to generate the strain YY7. Similarly, the two gene expression cassettes for a modified human reductase, ΔhRDM, and human cytochrome b5 were

introduced at the *LEU2* and *TRP1* chromosomal loci of BC300 to generate the strain YAB79.

The strain BC300 is defective in the five nutritional marker genes, *LEU2, HIS3, ADE2, TRP1* and *URA3*, a functional copy of each being essential for growth in minimal medium. These individual genes allow synthesis of the amino acids leucine, histidine and tryptophan, the nucleoside uracil and the nucleoside base adenine. This means that because there are inactivating mutations in the *LEU2, HIS3, ADE2, TRP1, URA3* genes, BC300 cells can only grow in minimal medium to which leucine, histidine, adenine, tryptophan and uracil are added. Therefore, selectable markers are essential elements for any yeast transformation procedure. For example, a yeast vector (i.e. plasmid), that contains the desired *CYP* gene and has the functional *URA3* gene as a selectable marker, has been transformed into BC300. Those cells which grow on minimal medium plates that contain leucine, histidine, adenine, tryptophan but lack uracil must contain the yeast plasmid. The functional *URA3* gene on the vector complements the *URA3* gene defect (i.e. *ura3*) in the cell and thereby restores cell growth in the absence of uracil.

One can use similar strategies to introduce multiple genes in a relatively stable manner using the other 'selectable markers' *LEU2, HIS3, ADE2* or *TRP1*. And that is how a gene copy of the modified NADPH P450 reductase (CPR; ΔhRDM) or the cytochrome b5 protein (substrate of cytochrome b5 reductase, CBR, which exists within yeast) can be co-introduced into the BC300 cells.

In addition, selection of yeast transformaants using a suitable antibiotic resistance gene, as a marker, has also been used for: (a) introducing new genes into the yeast genome and (b) disrupting target genes in yeast (Hashida-Okado 1998).

1.17.4 Yeast vectors

A wide range of vectors are available to meet various requirements for insertion, deletion, alteration and expression of genes in yeast. Most plasmids used for yeast studies are shuttle vectors, which contain sequences permitting them to be selected and propagated in *E. coli*, thus allowing for convenient amplification and subsequent alteration *in vitro.* The yeast vectors used in this project originate from the bacterial vector pBluescript and contain (a) pBluescript's bacterial origin of replication (ColE1ori) that can promote high copy number maintenance in *E. coli* and (b) the selectable antibiotic marker, the β-lactamase gene (*bla* or *Amp-r*) that confers resistance to ampicillin. The bacterial vector pBR322 derived yeast vectors have relatively low copy numbers and can confer resistance to either ampicillin (because of the presence of the *Amp-R* gene) or tetracycline (because of the presence of the *Tet-R*, tetracycline resistance gene) depending on the way that the yeast vector was constructed.

In addition, as mentioned before, all yeast vectors (Table 1.4) contain markers that allow selection of transformants containing the desired plasmid. They either bear an auxotrophic selectable marker or a drug resistant selectable marker.

Table 1.3. Components of the commonly used yeast plasmid vectors.

Plasmid	***YIp***	***YEp***	***YRp***	**YCp**
E. coli genes or segments				
Ori; bla; tet	+	+	+	+
Yeast genes or segments				
URA3; *HIS3*; *LEU2*; *TRP1*; *LYS2*; etc.	+	+	+	+
2 µm; 2µm-*ori REP3*	0	+	0	0
ARS1; *ARS2*; *ARS3*; etc.	0	0	+	+
CEN3; *CEN4*; *CEN11*; etc.	0	0	0	+
Host (yeast) markers				
ura33-52; *his3-Δ1*; *leu2-Δ1*; *trp1-Δ1*; *lys2-Δ201*; etc.	+	+	+	+
Stability	+++	+	+	+

1.17.4.1 YIp vectors

The YIp integrative vectors do not replicate autonomously, but integrate into the genome at low frequencies by homologous recombination. The site of integration can be targeted by cutting the yeast segment in the YIp plasmid with a restriction endonuclease and transforming the yeast strain with the linearized plasmid. The linear ends are recombinogenic and allow direct integration to the site in the genome that is homologous to these ends. In addition, linearization increases the efficiency of integrative transformation from 10 to 50 fold. Once integrated, the transforming DNA is part of the chromosome and segregate in mitosis and meiosis with the same high fidelity as a chromosome. Plasmids used for integration have a yeast selectable marker, but no other yeast elements (Sikorski 1989; Funk 2002). Strains transformed with YIp plasmids

(Table 1.4) are extremely stable, even in the absence of selective pressure. However, plasmid loss can occur at approximately 10^{-3} to 10^{-4} frequencies by homologous recombination between tandemly repeated DNA (if there be any in such sequences), leading to looping out of the vector sequence.

1.17.4.2 YEp vectors

The YEp yeast episomal plasmid vectors replicate autonomously in yeast because of the presence of a segment of the yeast 2 μm (μ) plasmid that serves as an origin of replication (2μ *ori*). The 2μ *ori* is responsible for high copy-number and high frequency of transformation of YEp vectors (Murray 1983; Sikorski 1989).

YEp vectors contain either a full copy of the 2μ plasmid, or, as with most of these kinds of vectors, a region which encompasses the *ori* and the *REP3* gene. The *REP3* gene is required in *cis* to the *ori* for mediating the action of trans-acting *REP1* and *REP2* genes which encode products that promote partitioning of the plasmid between cells at division. Therefore, the YEp plasmids containing the region encompassing only *ori* and *REP3* must be propagated in cir^{+} (i.e. circle-positive) hosts containing the native 2μ plasmid which provides the trans-acting *REP1* and *REP2* genes (Sherman et al., 1991).

Most YEp plasmids are relatively unstable under non-selective growth conditions, being lost in approximately 10^{-2} (i.e. 1 in 100) or more cells after ten generations of growth (each generation lasting around 90 min). Even under conditions of selective growth, only 60% to 95% of cells retain the YEp plasmid. The copy number of YEp plasmids ranges from 10-200 per cell of cir° hosts. However, the plasmids are not equally distributed among the cells, and there is a high variance in the copy number per cell in populations.

In this project, I have engineered the cir° yeast cells, from the strain BC300, which already contain stably integrated copy/copies of a modified human reductase (ΔhRDM) and/or cytochrome b5 to take up

(a) A YEp plasmid which encodes a variety of different human *CYP* genes, so that a CYP and its activating subunit (CPR; ΔhRDM), with or without cytochrome *b5*, will simultaneously be expressed within the same yeast cells, and

(b) One or two copies of YIp plasmids encoding human *CYP* genes, so that a CYP and its activating subunit (CPR; ΔhRDM), with or without cytochrome b5, will simultaneously be expressed within the same yeast cells.

1.17.4.3 YRp vectors

YRp vectors, containing ARS but lacking functional CEN elements, can be transformed into yeast at high frequencies. But these vectors are lost at too high a frequency, over 10% per generation, making them undesirable as vectors of choice (Sikorski et al., 1989; Sherman et al., 1991).

1.17.4.4 YCp vectors

The YCp yeast centromere plasmid vectors are autonomously replicating vectors containing centromere sequences, *CEN*, and autonomously replicating sequences, *ARS*. The YCp vectors are typically present at very low copy numbers, usually 1 per cell and are lost in approximately 10^{-2} cells per generation without any selective pressure. In most instances, the YCp vectors segregate to two of the four ascospores from an ascus, indicating that they mimic the behaviour of chromosomes during meiosis, as well as

during mitosis. The *ARS* sequences are believed to correspond to the natural replication origin of yeast chromosomes, and all share a specific consensus sequence. The *CEN* function is dependent on three conserved domains, designated I, II, and III; all three elements are required for mitotic stabilization of YCp vectors.

The stability and low copy-number of YCp vectors make them ideal as cloning vectors, for construction of yeast genomic DNA libraries, and for investigating the function of genes altered *in vivo*. *ARS1*, which is in close proximity to *TRP1*, is the most commonly used *ARS* element for YCp vectors, although others have been used. *CEN3*, *CEN4* and *CEN11* are the commonly used centromeres that can be conveniently introduced into YCp vectors.

1.18 Vector systems for heterologous expression of proteins in *Saccharomyces cerevisiae*

The yeast episomal plasmid, pSYE263 (created in Professor Chaudhuri's laboratory), contains a sequence that would allow autonomous replication in yeast. It was derived from yeast's endogenous 2µ-plasmid and contains an origin of replication which could promote high copy number if the gene expressed from the plasmid was non-toxic. Episomal 2µ-based plasmids are known to have fluctuating copy numbers depending on the gene that is expressed. Gene expression from pSYE263 is driven by the yeast *ADH2* promoter and contains the *URA3* gene as a marker for selection of yeast transformants in minimal medium that lacks uracil.

The yeast integrating plasmids do not contain the 2µ sequence but contain only one of the yeast genes *ADE2*, *HIS3*, *LEU2*, *TRP1* or *URA3* as a selection marker for growth of yeast

transformants in minimal medium that lacks adenine, histidine, leucine, tryptophan or uracil. New yeast integrating plasmids (Yip-s), which contain the *ADH2* promoter and which encode *ADE2, HIS3, TRP1* genes, were constructed by me for this project and are described in later Chapters of this report.

1.19 Prologue to the contents of this thesis

The aim of my investigation was to devise new systems for expression of human cytochrome P450 enzymes (CYPs) in baker's yeast, so that they are produced, as 'intracellular membrane bound' proteins, in high yields and activity. It is essential that CYPs bind to intracellular membranes to manifest activity. The goals were to find out the effects on 'activity' and 'yields' of CYP enzymes with the use of (1) human *CYP* genes (~1500 bps in length) which are 'chemically synthesised' using 'yeast biased codons', and (2) a non-toxic variant of the human P450 reductase gene (ΔhRDM; ~2000 bps; Patent WO2007129050A3) which had been genetically engineered before this work began. This was to test the concept that 'codon usage' is a crucial factor in expression of inherently toxic proteins.

A large number of plasmids (~100) have been constructed during the course of conducting the work described in this thesis. In the experimental Chapters 3 to 7, the construction of some of these plasmids and their applications will be discussed in detail.

Chapter 3 compares the expression of *CYP* genes, chemically synthesized using yeast biased codons, with the expression of native *CYP* genes isolated from a human liver

cDNA library. Expression of both types of genes, synthetic and native, was from an episomal plasmid.

Chapter 4 compares the expression of the synthetic human *CYP* genes from (a) an episomal plasmid with (b) single copies integrated at different sites of the yeast genome.

Chapter 5 describes the consequence of expressing synthetic human *CYP* genes from two separate copies integrated at two different sites of the yeast genome. Activities of microsomal enzymes, isolated from cells expressing two copies of the synthetic genes, have been compared with commercially available microsomal enzymes that are sold worldwide.

Chapter 6 describes the use of whole cells, expressing synthetic human *CYP* genes, for highly efficient biotransformation reactions of a starting chemical to a product or products.

Chapter 7 provides details of the significance of co-expressing two or three different CYP enzymes, from synthetic genes, within the same cohort of cells, in drug metabolism studies.

In summary, unique platform systems, based on bakers' yeast, have been devised that allows construction of 'stable' yeast strains that permit production of levels of highly active human CYP enzymes that has never been reported before.

Chapter 2 Materials and Methods

2.1 Chemicals, common reagents and equipment

Chemicals

All chemicals used were analytical and molecular grade. New England Biolabs, Sigma Chemicals, Fisher, Invitrogen Life Technologies, Becton Dickinson, Melford Laboratories, Roche and Bio-Rad UK were the main suppliers.

Water

All solutions used were made from Ultra-pure (18-Milli-Q) water obtained from a pure water system (Mllipore Q synthesis A-10, Millipore). For all yeast cell culture experiments, the media used were made from pure water and were autoclaved at 121°C, 15-psi pressure for 15 min.

2.2 Common reagents, solutions and media

TEG

50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 7.5.

50X TAE

242 g Tris base, 57.1 ml glacial acetic acid, 18.6 g EDTA, made up to 1 L with pure water.

10X TE

100 mM Tris-HCl, 100 mM EDTA.

LB Broth

A pre-mixed stock powder consisting of 10 g tryptone, 5 g yeast extract, 10 g NaCl, in 1 L pure water.

YP medium

1% Bacto-yeast extract, 2% Bacto-peptone.

YPD Broth

A pre-mixed powder from Difco (Cat No 242820), consisting of 1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose.

Synthetic Dextrose minimal medium (SD)

0.67% Bacto-yeast nitrogen base without any additional supplements, 2% glucose.

Supplemented minimal medium (SMM)

A SD medium that contains nutrients, depending on the auxotrophic markers required for a particular strain.

2.3 Equipment

The pieces of equipment used were the ones commonly used in a molecular biology/ cell biology laboratory which include:

Table 2.1. Equipment list.

Equipment	*Supplier*	*Model / Catalogue Number*
-20ºC Freezer	Sadia	Sadia 310
4ºC Fridge	Sadia	Sadia 310
Autoclave machine	Fisher Scientific	Classic 2100
Balance	Fisher Scientific	Op-1500
Bench-top Centrifuge	SANYO	MSE Micro-Centaur
Centrifuge (JA-17)	Beckman Coulter	Avanti™ J-20 XP
Dispensing Pipettor	Fisher Scientific	Eppendorf Mulltipette 2-10000µl
Double distilled water supply	Millipore	Milli-Q synthesis A10
Fluorescence microscope	Olympus	BX51
Fluorescence spectrophotometer	Shimadzu	UV-2401PC
Heated incubator	Fisher Scientific	Sanyo, MIR-163
Incubator 30ºC	Fisher Scientific	Sanyo, INA-320-010Y/MIR-162
Magnetic Stirrer/Heater	Fisher Scientific	IKA, SWT-750-030A
Microscope	Olympus	CKX41
Micro centrifuge MSE	Fisher Scientific	Sanyo, CEK-113-010P
Microwave oven	SANYO	800w compact microwave
PCR machine	Anachem	Biometra thermocycler, T3 Combi
pH meter	Mettler Toledo	Severn Multi
Sonicator	Hielscher	UP50H
Thermo mixer	Fisher Scientific	Eppendorf, BLD-455-010C
UV Illumination	UVP	C-80
Ultracentrifuge	Beckman Coulter	Optima L-100 XP
Ultra-low (-80ºC) freezer	Triple Red	Nuaire, NU6512E
Vortex mixer	Fisher Scientific	IKA, MPR-550-010H
Water Bath	Fisher Scientific	TYP E DMU 5
Ice machine	Scotsman	AF100

2.4 Basic Molecular Biology Techniques

Molecular techniques were important in every aspect of this project. Coding sequences of the human CYPs were chemically synthesized using yeast-biased codons. The CYPs that were synthesized were:

1) CYP1A1,
2) CYP1A2,
3) CYP1B1,
4) CYP2A6,
5) CYP2B6,
6) CYP2C8,
7) CYP2C9,
8) CYP2C9 Arg (R144C SNP),
9) CYP2C9 Ile (I359L SNP),
10) CY2C18,
11) CYP2C19,
12) CYP2D6_variant 1 (CYP2D6-1; Val^{374}),
13) CYP2D6_variant 2 (CYP2D6-2; Met^{374}),

14) CYP2D6_SNP2 (CYP2D6_*2),

15) CYP2D6_SNP10 (CYP2D6_*10),

16) CYP2D6_SNP39 (CYP2D6_*39),

17) CYP2R1,

18) CYP3A4,

19) CYP3A4_variant 2 (CYP3A4-2),

20) CYP2J2,

21) CYP4F3,

22) CYP4F2,

23) CYP4A11,

24) CYP7A1,

25) CYP17A1

26) CYP19A1.

At first, 80-100 base DNA fragments (Genewiz, USA) of each gene were chemically synthesized. The DNA fragments were then assembled using PCR with the help of a high-fidelity DNA polymerase. The amplified CYP coding sequences were cloned in a basic bacterial vector, pUC57 (Thermo Fisher Scientific), and the resultant plasmids were used for the work described in this report.

The plasmids were (a) digested with appropriate restriction enzymes, (b) the digests were loaded on to agarose gels, (c) the vector and the gene inserts were fractionated, (d) the inserts were isolated from agarose gel pieces, and (d) purified for further cloning in a yeast expression vector. After the genes were cloned into the yeast vector, the resultant plasmids were isolated using the alkaline lysis protocol (please see below). After that, pure plasmid DNA was isolate on commercially available silica-gel columns.

Yeast expression plasmids, encoding chemically synthesized *CYP* genes, were transformed (transferred) into yeast cells that contained a modified (non-toxic) mutant of the human CPR (ΔhRDM), and (in some cases) cytochrome *b5*. The transformed yeast cells (i.e. yeast strains) had the potential to produce active human CYP enzymes.

2.4.1 Molecular Cloning

2.4.1.1 Preparation of bacterial competent cells using CaCl2

DNA manipulation generally requires good competent bacteria. The *E. coli* DH5α strain is widely used in cloning applications due to its reliability, stability and improved quality of plasmid DNA obtained from mini preparations (mini-preps). The chemistry involved in the uptake of DNA by the cell is not yet fully understood. The cell is enabled to take up the circular DNA after treatment with cold calcium chloride ($CaCl_2$). The method was first described by Mandel and Higa (1970) and over the years has undergone many modifications.

Preparation of bacterial competent cells involved (a) streaking out DH5α cells from a glycerol stock on to LB agar plates (Sigma-Aldrich, Cat No L3147) that contained no antibiotic (i.e. ampicillin), (b) incubating plated cells over-night at 37°C, (c) picking an individual colony and inoculating it into a 5 ml LB broth (Sigma-Aldrich, Cat No L3147) contained in a 25 ml conical flask, and (d) growing cells over-night in an orbital incubator (shaking at 37°C, 220 rpm).

200 µl of cells from the overnight culture was added to 200 ml of LB broth that contained 1 ml of 50% glucose and cells were grown for about 4-5 h until OD_{600} (i.e. absorbance of cell suspension at 600 nm) reached 0.3-0.4. The culture was left for 10 min on ice. The centrifuge (Beckman Coulter Avanti™ J-17 XP) was chilled and the cells were centrifuged at 3500 rpm for 15 min at 4°C; the supernatant was discarded and the pellet was gently re-suspended in ice-cold 20 ml $MgCl_2$ (0.1M) and incubated for 20 min on ice to soften the cell wall. The cells were then centrifuged for 15 min, the supernatant was discarded and re-suspended in ice-cold 20 ml $CaCl_2$ (100 mM), incubated for 20 min and finally centrifuged for another 15 min when all supernatant was removed. The pellets obtained were gently re-suspended in 6 ml ice-cold $CaCl_2$ (100 mM) which contains 15% glycerol. 100 µl of cell suspension was aliquoted into Eppendorf tubes which were flash frozen in liquid nitrogen and stored at minus 80°C.

2.4.1.2 Construction of recombinant DNA molecules

One of the main aims of the project was to devise new systems for expression of human CYP enzymes in baker's yeast.

Within this aim, the first goal was to find out how 'chemically synthesized' human *CYP* genes, with codons that are yeast-biased, fare against CYP cDNAs isolated from a human liver cDNA library in terms of (a) 'CYP enzyme activity' and (b) 'CYP yield'.

For the above comparative analyses, 17 human *CYP* genes (13 of which are widely used for Drug Metabolism studies, and 4 which have crucial roles in the synthesis of steroids that are essential for human cells) were chosen. All the genes were synthesized using yeast-biased codons and were cloned into the basic vector, pUC57.

The pUC57 plasmids, bearing 'synthetic' *CYP* genes, were amplified by transforming into bacterial DH5α cells. The *CYP* genes were isolated from the pUC57 plasmids using restriction enzymes that cut at specific recognition sites at the 5' and 3' ends of the genes. The *CYP* genes were then ligated into a shuttle vector of choice (i.e. pSYE263) which has the ability to replicate inside both bacterial and yeast cells.

2.4.1.3 Plasmid vectors

One of the yeast vectors used, in the construction of recombinant DNA for yeast expression studies in this project, was the episomal 2μ-based vector pSYE263 (Figure 2.1). It contains the promoter from the yeast alcohol dehydrogenase gene 2, *ADH2*, which is one of the two alcohol dehydrogenase genes of yeast. Alcohol dehydrogenases are responsible for oxidation of ethanol to enable its removal from living cells.

The vector pSYE263 was already available in the lab and a small aliquot (10 µl) of bacterial stock from -80°C was inoculated into LB broth, which was grown overnight at 37°C and the plasmid was then isolated. Another plasmid used in this study was pBluKS(+)/ADH2p (Figure 2.2), which contained the *ADH2* promoter fragment (Saccharomyces Genome Database; YMR303C). The promoter DNA was isolated from this plasmid by growing cells from bacterial stocks, kept at minus 80°C, to construct a set of yeast integrative vectors for cloning *CYP* genes. These vectors permit integration of *CYP* gene expression cassettes into yeast cells' different chromosomal loci on the genome.

Both the plasmids (Figures 2.1 and 2.2) bear a β-lactamase gene which confers resistance to ampicillin. The restriction sites, that are depicted in the Figures, occur only once in the plasmids.

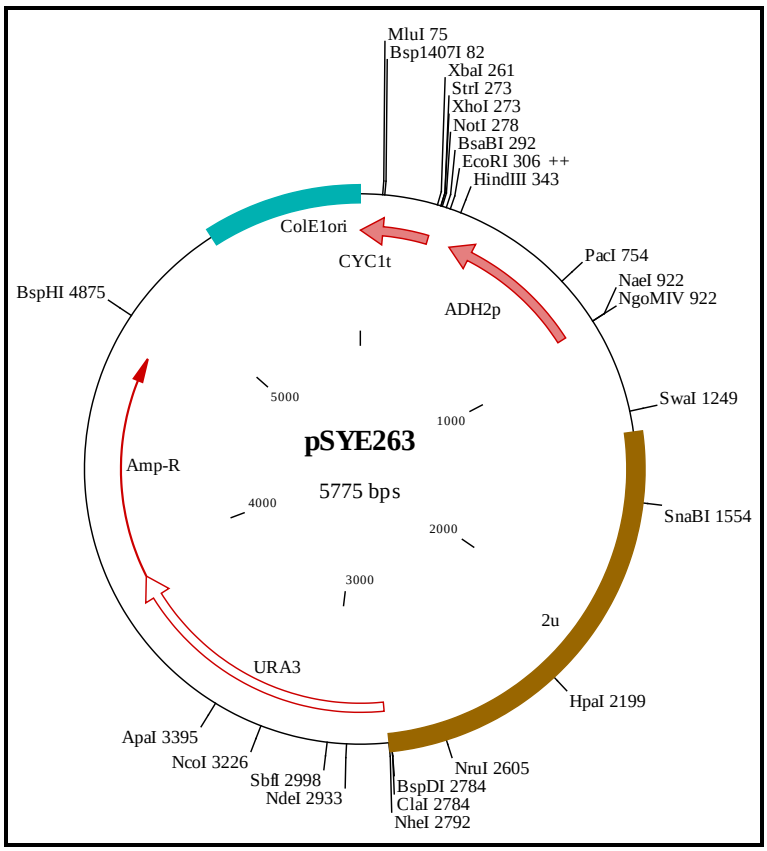


Figure 2.1. Map of the plasmid pSYE263, a 2µ-based episomal plasmid, which contains the yeast ADH2 promoter for inducible expression in yeast. The restriction sites that are shown occur in the plasmid only once.

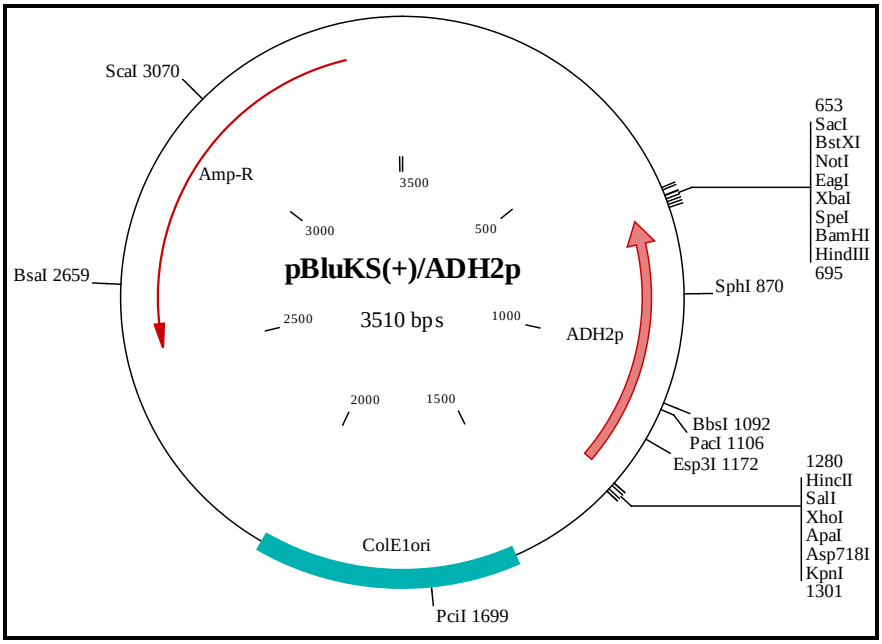


Figure 2.1. Map of the basic plasmid that contains the yeast *ADH2* promoter which was isolated from this plasmid for further cloning in yeast integrative vectors. The restriction sites that are shown occur in the plasmid only once.

2.4.1.4 Restriction enzyme digests

To be able to clone a DNA insert into a cloning or expression vector, both insert and vector have to be treated with restriction enzymes that create compatible ends. It is preferable that at least one of the enzymes used creates a sticky end to ensure that the insert is incorporated in the correct orientation.

All restriction enzymes used for this report were purchased from New England Biolabs, UK; they were supplied with their appropriate buffers. Amongst all the restriction digests carried out, only the *Xba*l site (TCTAGA) was found to be methylated which results in the site being resistant to cleavage. This occurs when the recognition sequence is preceded by GA or followed by TC. Hence, plasmids were passed through a dam-minus bacterial strain (SCS170) to prevent methylation, so that the site could be digested again with *Xba*I.

Coding sequence of all the genes began with an ATG (coding for Methionine, the Start site) and ended with a Stop codon (TAA, TAG or TGA). A restriction site (*BamH*I or *Bgl*II) preceded the ATG and a restriction site (*Xba*I or *Xho*I) followed the Stop codon.

2.4.1.5 Ligation

This involves the ligation of the insert to the linearized vector. It comprises the formation of covalent phosphodiester bonds between the 5' nucleoside phosphates of one fragment with the 3'-hydroxyl residue of another. The formation of the bonds can be catalysed by two different ligases, (a) bacterial DNA ligase or (b) bacteriophage T4 DNA ligase. The

latter (New England Biolabs, Cat No M0202S) was used because it also helps to join blunt-ended DNA fragments.

The ratio of amounts of insert to vector fragments ratio used is critical for high efficiency ligation. Powell (2001) calculates the ratio as:

$$\frac{\text{(ng of vector) x (kb size of insert)}}{\text{Kb size of vector}} \times \text{molar ratio of insert/vector} = \text{ng of insert}$$

The total reaction volume used for ligation was 20 µl and consisted of:

2µl	10x ligase buffer
2µl	Digested vector DNA (50 ng/µl)
	Equivalent amount of insert DNA
0.5 µl	T4 DNA ligase (20 NEB units/µl
	Sterile water to volume of 20 µl

Ligase was added last and gently mixed. A ligation mixture was incubated at 19°C (i.e. room temperature) on the bench, or 4°C overnight (in a cold room or a refrigerator). The reaction mixture was then used directly for transformation in competent cells (mostly DH5α, sometimes JM109). Through ligation, a new recombinant plasmid was created.

2.4.1.6 Transformation of ligation mixtures in $CaCl_2$ competent bacterial cells

The process of introducing foreign recombinant DNA into living cells is called transformation or transfection.

Competent cells are thawed on ice for 10 min. 5 ng of DNA from ligation mixture was introduced to the thawed competent cells and incubated for 30 min on an ice bucket. The

mixture was then heat-shocked at 42°C for 45 sec, and immediately transferred back to ice for recovery for 2 min. 800 µl of LB broth was added and incubated, shaken for 1 h at 37°C.

LB agar containing ampicillin (100 µg/ml, Fisher BioReagent, Cat No BPE1760-5) were poured on10 cm-diameter sterile plastic dishes and allowed to set. After 1 h, 50 µl of the cells from the transformation mixture were spread on the LB agar plates which contained ampicillin. The plates were incubated overnight at 37°C. The cells which grew on the plate contained plasmids that bear the β-lactamase (ampicillin resistant) gene.

2.4.1.7 Isolation and purification of recombinant plasmid DNA from transformants (mini preparations)

After the recombinant DNA was constructed via ligation, the clones were identified and confirmed through restriction enzyme digests. The cells, bearing the plasmid DNA (i.e. transformants), were then amplified through growth of bacterial cells (i.e. bacterial cell division). There were two types of plasmid DNA which were prepared in this study:

1. *Crude* DNA which were obtained via alkaline lysis preparations, and
2. *Pure* DNA which were obtained using commercially available mini silica-gel columns.

The alkaline lysis method was usually used to obtain relatively large amounts of DNA while the columns (commercially available from Qiagen or York Bio) have an advantage of yielding plasmids with reduced RNA which can accurately be quantified (i.e. concentrations of DNA can be determined in µg/µl) and stored longer.

The plasmids obtained by either of the two methods were used to

(1) Confirm that the insert and vector have the correct sizes, and that the plasmid has the correct restriction patterns after restriction enzyme digests.

(2) Transform yeast cells so that yeast transformants could be grown in cell culture and analysed for expression of active CYP enzymes.

2.4.1.8 Plasmid DNA preparation via alkaline lysis (crude preparation)

For this purpose, usually 4 individual bacterial colonies were picked from an LB agar ampicillin plate and inoculated into 5 ml LB broth containing 5 µl ampicillin (100 µg/ml). The cultures were grown overnight at 37°C, with shaking at 220 rpm inside an orbital shaker. The cultures were taken out and 1.5-3 ml of cells was spun down for 1 min at 13,000 rpm; the supernatants were discarded. The pellets were first re-suspended in Solution 1 and then the other two Solutions were added one after the other. They were:

(1) Solution 1 (4.5g glucose,12.5ml Tris-HCl pH 8, 10 ml EDTA pH 8, 4mg/ml RNase, made up to 500 ml with distilled water).

(2) Solution 2 (10 ml NaOH 10N, 50 ml 10% SDS, filled up to 500 ml with water); SDS is a detergent that lyses cells, whereas NaOH is a strong base that works to neutralize the strong acidity in Solution 3 to cause bacterial DNA to precipitate leaving the plasmid DNA in solution.

(3) Solution 3 (89 g potassium acetate, 58 ml acetic acid, filled up to 500 ml with distilled water.

100 µl of Solution 1 was used to re-suspend the pellet. Then 200 µl of Solution 2 was added and immediately placed on ice for 5 min. Then 150 µl of Solution 3 was added and inverted up and down six times, again incubated on ice for another 5 min; this neutralized the effect of Solution 2. The mixture was then spun down at the maximum speed 13,000 rpm for 10 min. Usually, a good pellet is formed from a slimy colourless solution that looks like starch. The supernatant was transferred into a new Eppendorf tube and 450 µl isopropanol was added, incubated for 10 min at room temperature. This was spun at 13,000 rpm for 10 min. The supernatant was discarded gently without disturbing the pellet and 500 µl of 70% ethanol was added and spun down for 8 min. The supernatant was removed gently and the pellet was air dried for 10-15 min at room temperature. 50 µl of 1xTE buffer was used to dissolve the dried pellet. Digest with restriction enzymes were performed on 2 µl of DNA to check the correct size of the insert DNA and authenticity of the newly created plasmid. The rest of the plasmid DNA was stored in a minus 20˚C freezer.

2.4.1.9 Preparation of pure plasmid DNA, using columns

Pure plasmids are prepared so that they could be stored for a longer period of time and the procedure followed was as specified by the manufacturer.

1.5-3 ml of overnight culture was spun down at 13,000 rpm for 1 min and the supernatant was discarded gently. With York Bio columns, there are three solutions that are used sequentially: Solution 1, Solution 2 and Solution 3. 100 µl of Solution 1 was added to

the pellet and re-suspended gently, 200 µl of Solution 2 was added immediately followed by 300 µl of Solution 3. The mixture was allowed to incubate for not more than 1 min and then centrifuged at 13,000 rpm for 10 min. The column was assembled inside the collection tube and the supernatant was transferred gently without disturbing the residue on the wall of the tube. 700 µl of washing solution containing 70% ethanol was added and spun down for 30 sec at 8,000 rpm; this step was repeated and the supernatant was discarded. The column was taken off and transferred into a new Eppendorf tube and eluted in 50 µl of nuclease free water (allowed to incubate for 2 min), spun down for 1 min at 13,000 rpm.

Digestions with restriction enzymes were performed using 1 µl (~4 µg) of plasmid DNA. The rest of the plasmid DNA was stored at minus 20˚C. After the plasmid was isolated, its concentration was determined by measuring absorbances at 260 nm and 280 nm in a 1 ml quartz cuvette with 1 cm path length. The ratio of the two absorbances gives the level of purity of the plasmid. Nucleic acids and proteins have absorbance maxima at 260 and 280 nm, respectively. Historically, the ratio of absorbances at these wavelengths has been used as a measure of purity in both nucleic acid and protein extractions. A ratio of ~1.8 is generally accepted as “pure” for DNA. High 260/280 purity ratios are not indicative of an issue whereas low values may indicate RNA/protein impurities.

The following formula was used to determine the concentration (amount) of DNA in µg/µl: OD260 X df* X 50/1000 = (DNA) µg/µl df*= dilution factor

2.4.1.10 Gel electrophoresis

After restriction digests, horizontal agarose gel electrophoresis was used to analyse the band size of insert DNA and restriction patterns of the plasmids. DNA fragments separate based on their molecular sizes. Since DNA is negatively charged and in solution it migrates to the positive pole in an electric field, higher the molecular weight of a linear DNA, the slower it migrates to the opposite end of the pole.

Gels are run for 20-25 min at 90 V, stopped and viewed under ultra-violet (UV) light. Presence of a fluorescent dye (Sybro Safe DNA Gel Stain, Life Technologies Cat No S33102) in the gel detects DNA bands in UV light at 260-310 nm. The gel composition may affect the visibility of the band size and its movement. Most gels were 1% w/v of agarose (Life Technologies, Cat No 15510027) in a 1 X TAE buffer (50x TAE, 40 mM Tris, 20 mM acetic acid and 1 mM EDTA, pH 8.4). The set gel was transferred into a tank containing the same electrolyte, 1X TAE, filled up to the maximum to cover the gel. Samples and DNA ladder (2–log DNA ladder; NEB, Cat No N3200L) were loaded (in 50% glycerol, 2% Ficol, 50 mM EDTA and 5% bromophenol blue in sterile water) onto the wells of the gel, and a potential difference was set to 90 V. After the gel ran for 30 min, it was taken out and viewed under UV light (BioRad, ChemDoc System).

A typical gel is depicted in Figure 2.3.

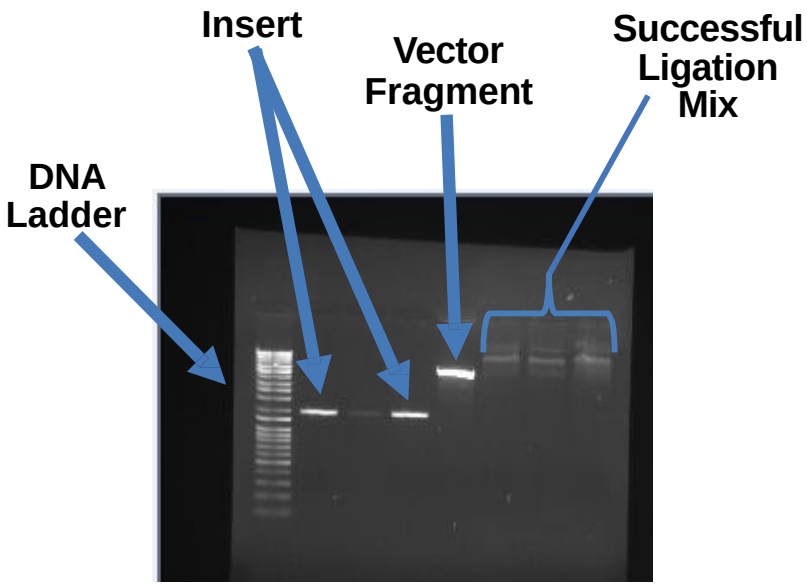


Figure 2.3. A typical agarose gel showing the different DNA fragments that were isolated to allow ligation between 'insert' and 'vector' fragments to obtain plasmids that were used for expression of CYPs.

2.4.1.11 Purification of DNA from agarose gels

Following gel electrophoresis, excised DNA fragments from gels were placed in a 1.5 ml Eppendorf tube, and DNA was isolated (using a kit from Promega; Wizard SV Gel and PCR Clean up System). 100 µl of membrane binding solution per 10 mg of gel slice was added and incubated at 65°C for 10 min on a Thermo-mixer (Fisher Scientific) shaking at 300 rpm until the gel completely dissolved in solution. The mini column was inserted into the collection tube and the dissolved gel was transferred, incubated for 1 min to allow the DNA to bind to the membrane. After centrifugation at 13,000 rpm for 1 min, the flow-through was discarded and the column was re-inserted into the collection tube, 700 µl membrane wash solution (containing ethanol) was added centrifuged at 13,000 rpm for 5 min and again the flow-through was discarded. The last step was repeated to ensure that a clean DNA fragment was obtained. The tube was centrifuged for 1 min at the same

speed and the column was transferred into a new Eppendorf tube. 50 µl of nuclease free water was added and incubated for 2 min at room temperature and centrifuged at maximum speed for 1 min. The flow through was checked by running a quick agarose gel to see the band fragment of 1 µl DNA. The isolated DNA was stored at -20°C until further use.

2.5 Yeast Molecular and Cell Biology

The yeast *Saccharomyces cerevisiae* was chosen as the heterologous host organism for the production of human CYP proteins. Yeast is a single-celled microbe that can be cultured and manipulated using techniques that have been published. Plasmids obtained from bacterial cells were introduced (via transformation) into yeast. The aim was to grow yeast transformants, containing the constructed plasmids which encoded different *CYP* genes, for production of active CYP enzymes using

a) Published general protocols and

b) More specific CYP-related protocols/ techniques that would be developed over the course of the work performed for this doctoral dissertation.

2.5.1 Yeast vectors

Some of the basic plasmids used in this study were available in the laboratory. In order to meet various requirements, such as for insertion, deletion, alteration and expression of genes in yeast, new plasmids were created during the course of this work.

The yeast vectors (both episomal and integrative) that were used in this study were derived from the basic plasmid pBluescript. This permitted selection on LB-agar plates that contained ampicillin. Since pBluescript contains the β-lactamase gene, bacterial cells that do grow in ampicillin must contain the yeast expression plasmid which was derived from pBluescript. Thus, ampicillin-resistant bacteria could also be grown in larger liquid cultures for amplification of plasmid DNA which could be used for further transformation into yeast.

The yeast episomal plasmid, pSYE263 contains a sequence that would allow autonomous replication in yeast. It was derived from yeast's endogenous 2μ-plasmid and contains an origin of replication which could promote high copy number provided the gene expressed from the plasmid was non-toxic. Episomal 2μ-based plasmids are known to have fluctuating copy numbers depending on the gene that is expressed. Gene expression from pSYE263 is driven by the yeast *ADH2* promoter and contains the *URA3* gene as a marker for selection of yeast transformants in minimal SD medium that lacks uracil.

The yeast integrating plasmids do not contain the 2μ sequence but contain only one of the yeast genes *ADE2*, *HIS3*, *LEU2*, *TRP1* or *URA3* as a selection marker for growth of yeast transformants in minimal medium that lacks adenine, histidine, leucine, tryptophan or uracil. New yeast integrating plasmids (YIp-s), which contain the *ADH2* promoter and which encode *ADE2*, *HIS3*, *TRP1* genes, were constructed by me for this project and are described later in the Results section.

2.5.2 Yeast media and culture conditions

The full medium (non-selective) YPD medium consists of 1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose. The mixture is available as a pre-mixed powder from Difco (Cat No 242820).

Synthetic (selective) SD minimal medium, used during this study, consisted of yeast nitrogen base without any amino acids. Different media were then prepared based on the supplementary nutrients (depending on the particular yeast strain's auxotrophic markers). The carbon source was glucose or raffinose (sugars that repress the *ADH2* promoter); mostly glucose has been used during the course of this thesis.

Exhaustion of glucose, through conversion of glucose to ethanol, induces the *ADH2* promoter and allows expression of CYP proteins. The stocks of all solutions and reagents (Table 2.2) were made sterile and stored under appropriate conditions.

Table 2.2. Nutrient concentrations for drop-out powders.

Nutrients	Final con. µg/ml *	Liquid stock	Sigma Aldrich Cat No
Adenine	40	500	A9126
L-Histidine	20	240	H8125
L-Leucine	60	720	L8000
L-Tryptophan	40	480	T0254
Uracil	20	240	U0750

* 8.3 ml/L of a stock solution that contained histidine, uracil, tryptophan or uracil, and 12.5 ml/L from an adenine stock solution were used. The tryptophan and histidine containing solutions were stored at 4°C.

2.5.3 Yeast strains and their manipulation

The yeast *S. cerevisiae* strains used during this research are derivatives of the basic strain W303-1a (MATa ade2-1 can-his-3-11, 15 leu2, 112 trp1-1 ura3-1) which is referred to in the lab as BC300. All yeast strains were freshly grown from a glycerol stock solution (which are stored at -80°C) before they are used for (a) transformation or (b) CYP expression studies. They were streaked out on a freshly prepared synthetic defined (SD) media with appropriate supplements and incubated at 30°C for 2-3 days. Single colonies were picked from the plates and inoculated into YPD liquid media. The culture was shaken overnight in an orbital shaker at 30°C, 220 rpm. The optical densities of cultures were measured at 600 nm (OD_{600}). This was done to monitor cell growth. Cells were also counted under a microscope, using a haemocytometer (Sigma-Aldrich, Cat No Z359629),

to check their viability before any further use. The haemocytometer chamber and the cover slip were sterilized and dried before use.

Cell suspensions (1 to 4 dilutions) were gently dropped on to the grid uniformly. Cells were counted within a 1 mm^2 ruled area which was divided into 25 squares centrally located within the chamber (Figure 2.4). Cells falling on the top or the right of the boundary were not counted but cells touching the left or bottom were all counted. Also all the yeast buds were counted as separate from their mother cells if they were one-half the size of their mother cells.

The total cell count was calculated as:

Total cell count/ml = [(total cell count x dilution factor)/number of squares] X 10000

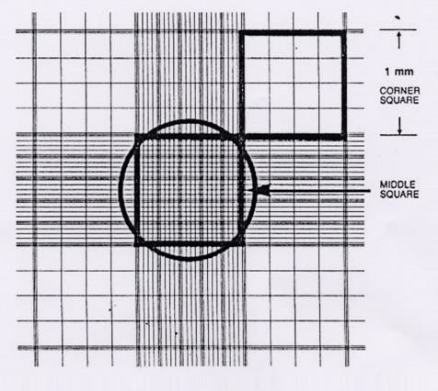


Figure 2.4: Standard haemocytometer chamber containing 9 square grids.

2.5.4 Yeast transformation with lithium acetate

The yeast *S. cerevisiae* has a very distinct way through which foreign DNA is introduced. The process involves the DNA attaching to the cell wall and entering the cell by endocytotic membrane invagination. One of the important elements for a successful yeast transformation is polyethylene glycol (PEG) which acts on the membrane to increase the transformation efficiency. The chemical lithium acetate and heat shock helps the DNA to pass through the cell wall. There are several ways to carry out yeast transformation but in this report, the lithium acetate (LiAc) method was adopted.

A yeast colony from a strain, streaked out on a fresh plate (as described above), was inoculated into 5 ml of liquid YPD yeast cell culture medium and incubated overnight in an orbital shaker at 30°C, with shaking at 220 rpm. The OD_{600} was determined after 16 h of growth and then 100 µl of the culture was added to fresh 100 ml of YPD in a separate flask. The cell titre should reach 2x 10^7 cells per ml after 3-5 h, after which the cells were harvested by centrifugation at 300 g for 5 min, washed twice in sterile water. 5X 10^7 cells was re- suspended in 1ml of 0.1 M LiAc. The carrier DNA (from salmon sperm) was boiled for 5 min and chilled on ice while harvesting the cells. The cell suspension was transferred into a new 1.5 ml Eppendorf tube, centrifuged for 1 min at 3000X g and the supernatant was discarded. The cell pellet was re-suspended in 1 ml of 0.1M LiAc and incubated for 10 min. 100 µl of cells were then transferred into a new 1.5 ml Eppendorf tube and centrifuged for 1 min. A master mix was prepared for yeast transformation (Table 2.3).

Table 2.3. Master mix for yeast transformation of DNA using lithium acetate.

PEG3500 50% w/v	250 µl
LiAc 1.0 M	36 µl
SS DNA	5 µl
DNA	3-5 µl
WATER	64 µl
TOTAL	360 µl

The items must be added one after the other as it appears on the Table.

After 360 µl of the mixture was prepared, it was mixed by vortexing for a few seconds and then it was incubated for 30 min at 30°C and transferred immediately for heat-shock to 42°C for another 30 min on water bath. The cells were then incubated on ice for 3 min and centrifuged for 2 min. The supernatant was discarded and 50 µl of sterile water was added to the cell pellet and the re-suspended cells were plated out on an SD plate to which appropriate supplements had been added. The plate was incubated at 30°C for 2-3 days. The transformants are picked and further streaked out on YP-glycerol (2%) plates to eliminate petites. The yeast S. cerevisiae is one of the very rare organisms that can exist without functional mitochondria and grow via a fermentative pathway. These mutant yeast cells are known as 'petites'. Petites may form, through the formation of free radicals, when a toxic protein is being expressed in yeast. Petites are slow growing and do not grow in glycerol which is a non-fermentative carbon source. Hence, the slow growing petite cells can be removed by growing yeast transformants in YP-glycerol.

2.5.5 Chromosomal integration of plasmids into yeast

Integrative plasmids are first linearized (by cutting the gene that is used as a nutritional selection marker in a way that at least 200 bps are in both 5' and 3' segments of the fragmented gene) before the DNA is introduced into yeast cells. The DNA then undergoes homologous recombination with the genomic DNA at the chromosomal locus of the linearized gene (i.e. the gene coding for the nutritional selection markers available in the strain BC300, that is, *ADE2*, *LEU2*, *HIS3*, *TRP1* or *URA3*).

2.5.6 Yeast live cell assays to measure CYP activities

The live yeast cell assays are rapid and relatively inexpensive compared to assays related to analysis of CYP microsomal activities. They are used to determine the expression levels of various CYPs via enzyme kinetic measurements. The assays were used to determine the expression levels of CYPs 1A1, 1A2, 1B1, 2D6(1), 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6(2), the 3 SNPs of 2D6 (*2, *10, *39), 2E1, 2J2, 3A4, 3A5, 4F3A, 17A1, and 19A1 produced from different expression plasmids created for this project.

2.5.7 Methods for live cell assays

The yeast strains containing the genes of interest were streaked out on minimal medium SD-agar plates with appropriate nutritional requirements. The plates were allowed to incubate for 2-3 days at 30°C. Three colonies were generally picked (to screen for high CYP producers often ~50 colonies were picked) and were grown according to the protocols available for expression of a particular CYP. The clones were cultured in

selective SW6 medium (SMM + glucose + casamino acids + nutrients required for auxotrophs) overnight at 30°C in an orbital incubator shaking at 220 rpm. On the next day, the OD_{600} of this pre- culture was recorded so that an appropriate number of cells could be seeded into full (non-selective) YPD medium. This main culture, to be used for expression of a CYP, was grown overnight for 18 h when cells were

(a) still in the exponential growth phase but

(b) at a time when all glucose in the medium was full exhausted.

Both aspects are important. Aspect (a) is essential for monitoring optimal levels of CYP activities; in stationary phase, CYP activities start decaying. Aspect (b) is crucial for the CYP protein to be produced at the highest levels; only after all glucose is converted to ethanol does the *ADH2* promoter allow full-blown expression (i.e. production) of a human CYP enzyme in yeast.

Cells from the main culture were counted so that a defined number of cells could be used for the fluorescence-based CYP activity assays.

2.6 Measuring CYP activities using a fluorescence plate reader

Cells grown up to the late exponential growth phase (16-20 h of incubation) were aliquoted into 1.5 ml Eppendorf tubes (labelled correctly) and centrifuged for 1 min on a bench-top centrifuge at 3,200 rpm. The pellets were then re-suspended in 500 µl 1xTE (50 mM Tris-HCl pH.7.4, 1 mM EDTA) and centrifuged for 1 min at the same speed as above. This was repeated twice. The supernatants were removed carefully and the pellets

were finally re-suspended in 450 µl of 1X TE. 50 µl of cell suspensions were transferred into a sterile 96-well microtiter plate to which 50 µl of a substrate mixture (depending on the substrate for a specific CYP) was added. The parameters for the fluorescence plate reader (Synergy HT BioTek) were set using the appropriate extinction/emission filters and appropriate gain sensitivity setting to obtain the best kinetic output from the plate reader (Table 2.4). The plate was incubated at 30°C for 30 min before the fluorescence emissions were measured.

Table 2.4. Excitation and Emission filters used in monitoring the amount of fluorescence emission.

Substrates	Excitation/Emission (nm)	Reference
CEC	410/460	White (1998)
Coumarin	355/460	Donato et al.(1998)
BFC	410/510	Butters et al. (1993)
DBF	485/520	Stresser et al. (2000)
BOMCC	410/460	Liu et al.(2010)
EROD	530/590	Afshin (2014)

2.6.1 Preparation of TE buffer (100 ml)

5 ml of 1 M Tris-HCl pH 7.4 + 200 µL of 0.5 M EDTA pH 7.5, the volume was topped up to 100 ml with water.

2.7 Bradford Assay

In 1976, Bradford developed a method for measuring protein concentrations which involves the binding of Coomassie brilliant blue G-250 dye to proteins. The dye exists in three forms: cationic, neutral green and anionic blue (Compton and Jones, 1985). Immediately after the dye binds to the protein(s), the dye changes from protonated red (A_{max}=470 nm) to stable un-protonated blue (A_{max}= 595 nm).

2.7.1 Dye reagent for Bradford assay

The protein standard was bovine serum (BSA) (Sigma-Aldrich, Cat No A6003). The concentration for BSA stock solution was 10 mg/ml to draw a standard curve (Table 2.5; Figure 2.6). The protein assay dye concentrate (Bio-Rad, Cat No 500-0006) contains 450 ml of dye solution, phosphoric acid, and methanol.

Initially, 4 µl of microsomes (see Section 2.8) were diluted in 800 µl of sterile water. This was used for making further dilutions. For example, for the CYP19A1 microsomes, two dilutions were made:

1 in 5 200 µl (from 4/800 stock) + 800 µl water

1 in 8 125 µl (from 4/800 stock) + 875 µl water

Table 2.5. For BSA standard curve, 0.1mg/ml BSA solution was prepared from the 10mg/ml stock solution.

Tube No.	1	2	3	4	5	6	7
Volume of BSA (0.1mg/ml)	0	25	50	100	150	200	250
Volume of H_20 in µl	1000	975	950	900	850	800	750
BSA amount in µg	0	0.5	1	2	3	4	5
BSA BioRad reagent added in µl	250	250	250	250	250	250	250

Different amounts of BSA (in a total volume of 150 µl) were added per well. End point absorbance was read at 590 nm. The BSA standard curve is shown in Figure 2.6.

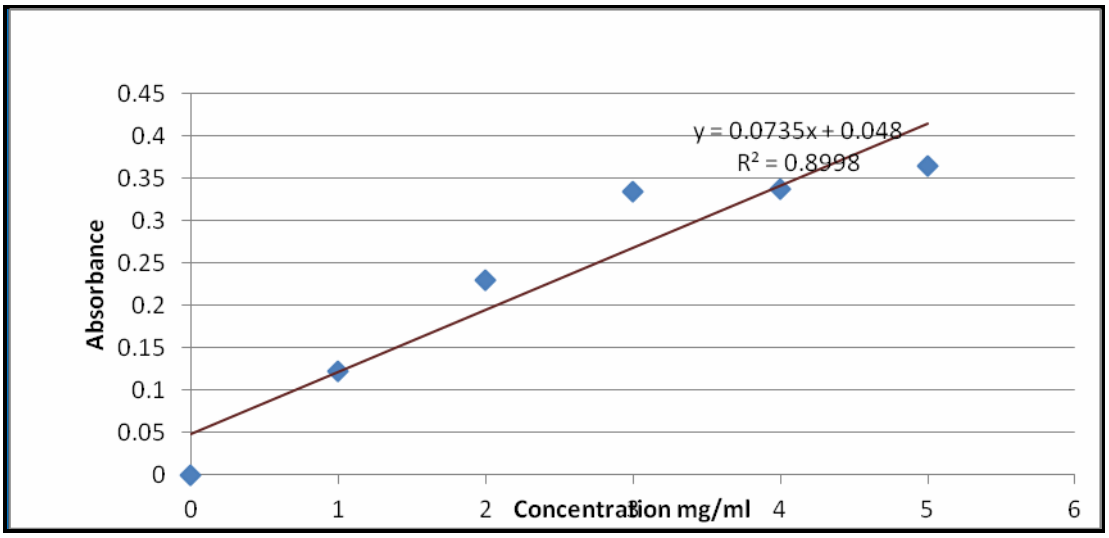


Figure 2.6. The BSA standard curve. The concentrations refer to the values in Table 3.

2.8 CYP microsome preparation from yeast cell cultures using PEG precipitation

For microsome preparations, the protocol that was used was similar to the one described by Mecellan et al. (2000), only the amounts of cells grown were scaled up. Depending on the amounts of microsomes required, the final volume of main culture was between 400 ml and 1600 ml. The cells were harvested washed with DMB-A buffer that contained a Serine protease inhibitor (0.266 mM DTT, 2.66 mM of AEBSF), and the cell pellets were stored at -80°C. The protease inhibitor AEBSF has been shown to inhibit trypsin, chymotrypsin, plasmin, kallikrein and thrombin. As an alternative to PMSF, AEBSF offers lower toxicity, improved solubility in water and improved stability in aqueous solutions.

2.8.1 Cell disruption

The protocol for preparation of microsomes used a combination of continuous cellular disruption under pressure, followed by differential centrifugation. The liberated membrane-bound CYP proteins were then concentrated using a combination of NaCl and PEG precipitation. The devised protocol has been adapted from the methodology detailed by McLellan *et al.* (2000).

Cell pellets were removed from the -80˚C freezer and kept on ice for 30 min. The cell pellet was re-suspended in DMB A buffer (to 1g of wet cell pellet, 1.4ml of DMB-A buffer was added). The cell suspension was centrifuged on a bench-top Sorvall centrifuge. ~35 g cell pellet was gently re-suspended (with the help of a glass rod) in 50 ml DMB-A buffer to which 1.33ml (100 mM AEBSF) and + 0.133ml (100 mM DTT) were added.

Ensuring homogeneity of cell suspension is essential for cell disruption. Failure to add AEBSF and DTT is likely to result in degraded CYP proteins.

Before the cell pellet was disrupted, the Constant Cell Disruption equipment (Constant Systems Basic Z model with continuous processing head) was chilled for 30 min at 4°C before use. Pressure was set to 22.5 KPSI (Kilo-Pound per Square Inch). Disruptor was washed with about 50 ml of DMB-A buffer to ensure that the system was operating correctly. Cell suspension was added into the cell disruptor gently. The cells were then processed in the cell disruptor.

The cell lysates obtained were collected from the cell disruptor and were centrifuged at 4,600 rpm (4,302 g) for 20 min at 4˚C using a Sorvall bench-top centrifuge [this was to remove all cell debris and broken cell membranes]. The resulting supernatants were then transferred into chilled 30 ml JA17 tubes for use in the high-speed Beckman Coulter JA 17 rotor in an Avanti J-20XP centrifuge and spun at 16,000 rpm (35,267X g) for 15 min at 4°C (this was to remove cell debris, nuclei, peroxisomes, lysosomes and mitochondria).

Percentage disruption was calculated by comparing the known cell pellet wet weight with that which remained after disruption. Centrifugation was repeated twice into freshly cooled JA17 tubes.

2.8.2 PEG3500 precipitation of cytosolic fraction performed at 4°C

The supernatants were transferred into large capacity chilled buckets for the high-speed Beckman Coulter JLA 10.5 rotor for centrifugation in an Avanti J-20XP centrifuge.

The supernatants were then diluted with ice cold DMB-TES buffer containing both DTT and AEBSF [0.4 mM AEBSF (4.0µg/ml), 0.04 mM DTT (0.4µg/ml)]. The bucket was then placed on a magnetic stirrer in the cold room and stirred at approximately 1-2 rpm. NaCl was then added to this mixture to give a final concentration of 0.125 M, and mixed gently.

The required volume of ice-cold 50% PEG solution was transferred into a separating funnel and added drop-wise (1-2 drops per second) to the above solution whilst gently stirring the solution.

2.8.2.1 An example of a typical microsome preparation

100 ml of supernatant diluted with 375 ml DMB-TES to which had 11.88 ml (5 M NaCl). To this mixture, 118.8 ml (50% PEG) was added drop-wise. .

After addition of PEG4000, the magnetic flea was removed from the bucket and the supernatant mixture was then left on ice in the cold room for 20 min, and then spun at 12,000X g, (8,034 rpm), for about 20 min at 4°C in the high-speed Beckman Coulter JLA 10.500 rotor in the Avanti J-20XP centrifuge. This step pelleted the microsomal fraction of the cells. The supernatant (cytosolic fraction) was poured off very gently. The surface of the pellet was washed twice with roughly the same volume of DMB-buffer B as that of the size of the pelle**t**.

The pellets were transferred (using long, thin spatulas) into a 15 ml homogeniser and re-suspended in DMB-buffer B. Depending on the size of pellet this volume varied. The pellets were very gently re-suspended by passing the plunger of the homogeniser up and

down four to five times avoiding excessive frothing. Microsomes were aliquoted out, snap-frozen using liquid nitrogen, and stored at -80˚C. A 50 µl aliquot of microsomes was used to check for amounts of CYPs produced using carbon monoxide (CO)-difference spectroscopy.

2.8.3 Reduced CO difference spectra for calculation of cytochrome P450 enzyme concentration in microsomes

Microsomes are turbid suspensions made up of several membrane-containing structures within a cell and are obtained after opening up the cell wall and isolating the intracellular membrane fraction. These suspensions are opaque to standard spectroscopy, because they scatter light so badly. The only way to measure a spectrum on a turbid sample like this was to make a special instrument with the light detector very close to the cuvette, and to use dual beams and perform difference spectroscopy. Cytochrome P450 is haemoprotein. When the haem Fe^{3+} is reduced to Fe^{2+} by the addition of sodium hydrosulfite and complexed with carbon monoxide, a characteristic absorption spectrum can be measured (Klingenberg, 1958). The carbon monoxide (CO) binding assay (Omura and Sato 1964) is a method for quantifying the amounts of CYP holoproteins (i.e. active proteins) in microsome preparations. The reduced, carbon monoxide complexed, difference spectrum of P450, has a maximum absorbance at 450 nm.

2.8.3.1 CO biding spectrum procedure

Difference spectra of microsomal preparations were measured in a spectrophotometer (Shimadzu, UV-2401PC) using disposal plastic cuvettes (Sarstedt, Cat. No. 67.742). 850 µl of 100 mM potassium phosphate and 20% glycerol, pH 7.5, was added in cuvette, and left for one min. Then a 'few grains'(usually 3-5 mg) of sodium hydrosulfite powder was added; mixed gently to prevent any bubble formation into the cuvette and then left for one min on the table at room temperature. 150 µl of microsomes was added into the cuvette and mixed gently. Two cuvettes (one containing buffer with sodium hydrosulfite without microsomes and another with microsomes were prepared and a baseline of light absorption of the buffer and microsome mixture was recorded in the dual-beam spectrophotometer from 400 nm to 500 nm. Carbon monoxide was bubbled slowly into one sample cuvette for about one min, 1 bubble/sec. Light absorption was recorded again from 400 nm to 500 nm. The concentration of cytochrome P450 in the cuvette was calculated from the absorption change at 450 nm relative to the absorbance change at 490 nm, using the formula below:

CYP content (nmole/ml) = $[(A_{450nm} - A_{490\ nm})$ X dilution factor X 1000]/ 91

Specific CYP content (nmole/ mg protein) = CYP content/ total protein in the reaction

Extinction coefficient (91 $mM^{-1}cm^{-1}$) is the fraction of light lost to scattering and absorption per unit distance in a participating medium. It is the sum of absorption coefficient and scattering coefficient.

2.8.3.2 Reagents and Media used for microsome preparations

All solutions were autoclaved unless otherwise stated.

DMB A Buffer

- 162.5ml (4M sorbitol) final conc 0.65M,
- 10ml (1M Tris-HCl pH 7.5) final conc 10mM,
- 200µl (0.5M EDTA pH 8.0) final conc 0.1mM, made up to 1 litre.

Microsome Buffer A

- 162.5ml of 4 M Sorbitol, final concentration 0.65 M,
- 10 ml of 1 M Tris pH 7.5, final concentration 10 mM,
- 200 µl of 0.5 M EDTA pH 8.0, final concentration 0.1 mM, made up to 1 litre

with MQ water and stored at 4°C.

Microsome Buffer C

- 1 ml (1 M Tris-HCl pH 7.5) final concentration 10 mM,
- 200 µl (0.5 M EDTA pH 8.0) final concentration 1.0 mM,
- 40 ml of 50% (v/v) glycerol final concentration 20%,

made up to 100 ml with MQ water and stored at 4°C.

100mM AEBSF [4-(2-Aminoethyl)-benzenesulfonyl fluoride, HCl], Protease Inhibitor Working Solution

239.7 mg of AEBSF (Melford, Cat No MB2003) was dissolved in 10 ml of MQ water and the solution was stored at -20°C. The solution is corrosive and causes burns.

DL-Dithiothreitol Working Solution (100 mM)

154.2 mg of dithiothreitol (Sigma, Cat No. D9779-SG) was dissolved in 10 ml MQ water. 500 µl aliquots were stored at -20°C.

Sutter's Buffer B

- 250 ml 4 M sorbitol, final concentration 2.0 M,
- 5 ml 1 M Tris-HCl pH 7.5, final concentration 10 mM,
- 100 µl 0.5 M EDTA pH 8.0, final concentration 0.1 mM,
- 500 µl 1.0 M $MgCl_2$, final concentration 1 mM made up to 500 ml.

Sutter's Buffer C

- 1 ml 1 M Tris-HCl pH 7.5, final concentration 10 mM
- 200 µl 0.5 M EDTA pH 8.0, final concentration 1.0 mM
- 40 ml 20% glycerol, (from 50% glycerol stock) made up to 100 ml.

Pefabloc (100mM)

239.5 mg of Pefabloc was in 10 ml of pure H_2O. 500 µl aliquots were stored at -20°C.

SD Min Agar (500 ml)

- 3.35 g YNB (BD Cat No MD21152),
- 10 g Glucose (Melford Cat No G1400),
- 7.5 g Agar (Oxoid Cat No LP0013),
- 1 pellet of NaOH (Fisher Cat No 359-500).

SW6 Min broth (SW6) (1 Litre)

- 6.7 g YNB BD Catalogue number: MD21152 from Becton Dickinson,
- 40 ml of a 50 % glucose solution Catalogue number: G1400 from Melford.

Buffer for Harvesting Cells (1 Litre)

- 118.6 g/L Sorbitol, final conc. 0.65 M (Melford Cat No S0807)
- 10 ml 1M Tris-HCl pH 7.5, final concentration 10 mM (Fisher Cat No BP152-1),
- 200 µl 0.5 M EDTA pH 8.0 final concentration 0.1 mM (Sigma Cat No E9984).

2.9 CYP assays

2.9.1 Stock solutions for all CYP assays

Solution A: 200 mg $NADP^+$ (Sigma-Aldrich Cat N^o N0505, MW 765.4), 200 mg D-glucose-6-phosphate disodium salt hydrate (Sigma-Aldrich Cat N^o F7250, MW 304.1), 1M $MgCl_2$ solution (Sigma-Aldrich Cat No M1028) were used to make a 10 ml solution in deionized water which gave final concentrations of 26.13 mM, 65.77 mM, and 65.42 mM of the three constituents. The solution was stored in aliquots at –20°C.

Solution B. 250 g U glucose-6-phosphate dehydrogenase (Sigma-Aldrich Cat N^o G6378) was dissolved in 6.25 ml solution of 5 mM trisbasic sodium citrate (Sigma-Aldrich Cat No S46410) and stored in aliquots at –20°C.

Solution C. Solution C was freshly prepared just before the assay by mixing 1.5 ml of 0.5 M KPO_4 pH 7.4, 1.5 ml of solution A and 0.3 ml of solution B. The final volume was made up to 15 ml with deionised water.

0.5 M Potassium Phosphate (KPO4) buffer pH 7.4 (Kpi buffer). 19.8 ml of buffer A and 80.2 ml of buffer B (see below) were mixed and the final volume was made up to 200 ml with deionised water and pH was adjusted to 7.4 (using 0.5 M HCl and 0.5 M NaOH).

Buffer A = 136.1 g KH_2PO_4/L (1 M), autoclaved.

Buffer B = 174.2 g K_2HPO_4/L (1 M), autoclaved.

0.1 M Potassium Phosphate (KPO_4) buffer, pH 7.4. 1.98 ml of buffer A and 8.02 ml of buffer B were mixed and the final volume was made up to 100 ml with deionised water and the pH was adjusted (using 0.5 M HCl and 0.5 M NaOH).

Buffer A = 136.1 g KH_2PO_4/L (1 M), autoclaved.

Buffer B = 174.2 g K_2HPO_4/L (1 M), autoclaved.

50 mM Tribasic Sodium Citrate. 147 mg of tribasic sodium citrate (Sigma-Aldrich Cat Nº S4641) was dissolved in deionized water and the final volume was made up to 10 ml.

2.9.2 Assay procedures

The substrates for enzymes assays used in this study are CYP isozyme-specific fluorogenic substrates which upon oxidative cleavage yield fluorescence at specific excitation-emission wavelength. The substrates are light sensitive. Therefore, all assays were set up in the laboratory with yellow lights switched on.

The reactions were performed in black 96-well plates with clear flat bottom (Fisher Cat No, FB86083). The assay plate and the solutions were pre-warmed at 37ºC before initiation of the assay. The plate layout, reaction temperature (37°C), reaction time (20 to 40 min), fluorescence sensitivity, the wavelength parameters and other kinetic assay parameters (i.e. excitation-emission wavelengths) were set as is indicated in the following chapters. Each reaction was performed in triplicate. Each 100 µl reaction contained a specific concentration of Kpi buffer (as indicated in respective chapters), solution C, CYP isozyme and an isozyme-specific fluorogenic substrate. Blank values were measured in reactions where CYP isozyme was not added. The known concentrations of metabolites

were used to plot standard curves. The fluorescence was measured in terms of relative fluorescence unit (RFU) in kinetic reactions over a period of 30 to 60 min. Finally, the CYP activities were calculated as product formed/ min/ pmole of CYP or product formed/ min/ ml.

2.9.2.1 EOMCC Assay

Kinetic studies with CYP2D6 and CYP2E1 microsomes (i.e. measurement of activities) were performed using the Vivid® fluorogenic substrate EOMCC (3-cyano-7-(ethoxymethoxy) coumarin) as per the standard Invitrogen Screening Kit protocol (Marks et al, 2002 and 2003). Human CYP2D6 and CYP2E1 de-methylate EOMCC to form the fluorescent product 3-cyano-7-hydroxycoumarin (CHC). The reactions were carried out in a reaction volume of 100 µl/ well in 96-well black plates (Fisher Scientific, Catalogue No FB86083). All reactions were performed at 37ºC in 100 mM potassium phosphate buffer pH 8.0, containing 10 µM EOMCC, 1 pmol (CYP2D6 or CYP2E1) and the NADPH regenerating system (1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, 0.4U/ml glucose-6-phosphate dehydrogenase). The fluorescent values of the product were recorded on the Biotek Synergy HT plate reader using an excitation wavelength of 410 nm (± 20 nm) and emission wavelength of 460 nm (± 50 nm). Changes in fluorescence were measured as relative fluorescence units (RFU). Kinetic progression of reactions was measured over 30 to 60 min.

2.9.2.2 CEC Assay

CYP1A2, 4F3A and CYP2C19 activities were measured using the fluorogenic substrate, 3-cyano-7-ethoxycoumarin (CEC). CEC is metabolised mainly by CYP2C19, CYP4F3A and CYP1A2 to a fluorescent product 3-cyano-7-hydroxycoumarin (CHC) (Donato et al. 2004). The reactions were carried out in a reaction volume of 100 µl/ well in 96-well black plates (Fisher Scientific, Catalogue No FB86083). The reactions were performed at 30ºC in 100 mM potassium phosphate buffer pH 7.4, containing 10µM CEC, 1 pmol CYP1A2, 4F3A or CYP2C19 and the NADPH regenerating system (1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, 0.4U/ml glucose-6-phosphate dehydrogenase). The fluorescent values were recorded on the Biotek Synergy HT plate reader using an excitation wavelength of 410 nm (± 20 nm) and emission wavelength of 460 nm (± 50 nm). Changes in fluorescence were measured as relative fluorescence units (RFU). Kinetic progression of reactions was measured over 30 to 60 min.

2.9.2.3 EROD Assay

The catalytic activities of CYP1A1 and CYP1B1 were measured using the ethoxyresorufin-O-deethylase (EROD) assay (Klotz et al. 1984). CYP1A1 and CYP1B1 convert 7-ethoxyresorufin (7-ER) to resorufin in the presence of NADPH and oxygen and the assay is known as the EROD assay. The amount of resorufin produced is measured fluorometrically and it reflects the level of 7-ethoxyresorufin o-deethylase activities of the two enzymes. Both CYP1B1 and CYP1A1 can catalyse de-ethylation of the substrate 7-ethoxyresorufin (7-ER) to form the product resorufin, which exhibits fluorescence at excitation wavelength of 530 nm and emission wavelength of 590 nm.

The production of the metabolite resorufin, by deethylation of the fluorogenic substrate 7-ethoxyresorufin was measured over 30 to 60 min. The assay was performed as described before (Klotz et al. 1984). The reaction kinetics was carried out at 30ºC in a reaction volume of 100 µl/ well in 96-well black plates with transparent flat bottom (Fisher, Catalogue No FB86083). The assay mixture contained 100 mM Potassium phosphate buffer pH 7.4, containing 5 µM 7-ethoxyresorufin, 1 pmole CYP1B1 or CYP1A1 and the NADPH regenerating system (1.3 mM NADP+, 3.3 mM G6P, 3.3 mM $MgCl_2$, 0.4U/ml G6PDH). Resorufin formation was measured fluorometrically on the Biotek Synegy HT plate reader (excitation wavelength of 528 ± 20 nm and emission wavelength of 590 ± 20 nm). Changes in fluorescence were recorded over 30 to 60 min as relative fluorescence units (RFU).

2.9.2.4 DBOMF Assay

CYP3A4, CYP2C8, and CYP3A5 activities were measured using DBOMF as the fluorogenic substrate. The production of the metabolite fluorescein, by dealkylation of Vivid® fluorogenic substrate dibenzyl-oxymethyl-fluorescein (DBOMF) was measured over 30 to 60 min at 30ºC. The assay was performed as per the standard Invitrogen Screening Kit protocol. The reactions were carried out at 30ºC in a reaction volume of 0.1 ml/well in 96-well black plates. The assay mixture contained 100 mM Potassium phosphate buffer pH 8.0, containing 4 µM DBOMF, 1.0 pmole CYP3A4, CYP2C8, or CYP3A5 and the NADPH regenerating system (1.3 mM NADP+, 3.3 mM G6P, 3.3 mM Mg Cl_2, 0.4U/ml G6PDH). Fluorescein formation was measured fluorometrically on the Biotek Synegy HT plate reader (excitation wavelength of 485 ± 20 nm and emission

wavelength of 528 ± 20 nm). Changes in fluorescence were recorded over 30 to 60 min as relative fluorescence units (RFU).

2.9.2.5 7-MFC Assay

CYP2C9 activity was measured using 7-MFC as the fluorogenic substrate. The production of the metabolite HFC, by dealkylation of Vivid® fluorogenic substrate 7-methoxy-4-(trifluoromethyl)-coumarin(7-MFC) was measured over 30 to 60 min at 30ºC. The assay was performed as per the standard Invitrogen Screening Kit protocol. The reactions were carried out at 30ºC in a reaction volume of 0.1 ml/well in 96-well black plates. The assay mixture contained 100 mM Potassium phosphate buffer pH 8.0, containing 50µM 7-MFC, 1.0 pmole CYP3A4, CYP2C8, or CYP3A5 and the NADPH regenerating system (1.3 mM NADP+, 3.3 mM G6P, 3.3 mM Mg Cl_2, 0.4U/ml G6PDH). HFC formation was measured fluorometrically on the Biotek Synegy HT plate reader (excitation wavelength of 431 ± 9 nm and emission wavelength of 535 ± 20 nm). Changes in fluorescence were recorded over 30 to 60 min as relative fluorescence units (RFU).

2.9.3 Inhibition of CYP enzymes bound to microsomes and expressed within whole cells

Cells were taken during the exponential growth phase at ~18 h of incubation. 4×10^8 cells were taken from the overnight culture and aliquoted into an Eppendorf tube (this is equivalent to approximately 25 ODs). These cells were then centrifuged in a bench top centrifuge for 1 min at 13,200 rpm (16.1X g). The supernatants were removed carefully.

The cell pellets were then re-suspended in 1000 µl of TE buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA). The re-suspended cells were centrifuged again at 13,200 rpm (16.1X g) for 1 min. The supernatants were one again removed by careful pipetting and again re-suspended in 900 µl TE buffer and centrifuged for 1 min at 13,200 rpm (16.1X g). This step was repeated once again. The supernatants were again removed by careful pipetting and the cell pellet was re-suspended in 900 µl of TE buffer bringing the end volume up to 1000 µl.

These cells were diluted further 1 in 10 before carrying out an IC_{50} assay with compound concentrations in serial dilutions.

2.9.3.1 Preparation of CYP inhibitors

For most inhibitors, a master stock at 400 µM concentration was prepared.

The working concentrations of inhibitor were obtained by diluting the stock in 1X TE buffer. The working concentrations were 4X stronger than the final concentration as only 25 µl of inhibitor solution was added to each well to obtain a final volume of 100 µL per well (Table 2.6).

Table 2.6. Concentrations of inhibitors used for IC50 determinations.

Working concentration (μM)	Final concentration in each well (μM)
120	**30**
40	**10**
12	**3**
4	**1**
1.2	**0.3**
0.4	**0.1**
0.12	**0.03**
0.04	**0.01**

2.9.3.2 Substrate mixture preparation

0.5 µL of substrate was added to 24.5 µl of TE buffer. 25 µl of substrate mix was added to each well of the plate. Final concentrations of substrates used are mentioned in the Figures of the Chapters that describe the experimental results.

2.9.3.3 General procedure for CYP inhibition studies

50 µl of cells was added to each well. 25 µl of each inhibitor concentration was added to three wells on the plate (triplicate). The plate was placed in the incubator at 30°C for 10 min. After the 10 min incubation, 25 µl of substrate was added to each well on the plate.

The plate was then placed in the BioTek plate reader and read for 30 to 60 min.

2.9.3.4 CYP inhibition assay parameters

The assay is carried out between time periods of 30 to 60 min using appropriate filters that would monitor extinction/emission of the fluorescent products formed. Appropriate gain sensitivity settings were used to obtain the best kinetic output from the Synergy HT BioTek plate reader. These parameters varied depending on the level of CYP expression within the recombinant yeast cells.

The plate reader is set up to shake the plate for 3 sec at an intensity of 4 (in a scale of 1 to 10) between each reading. Kinetic analysis is carried out at 30°C. Table 2.7 provides an example outlining the plate parameters for assaying CYP1A1 enzyme.

Table 2.7. An example outlining the parameters with which CYP1A1 enzyme was analysed in each yeast strain.

Enzyme	**Substrate**	**Excitation** (Bandwidth of filter)	**Emission** (Bandwidth of filter)	**Gain** (Top)	**Substrate Concn.** (µM)	**Buffer**
CYP1A1	7-ethoxyresorufin-deethylase	530 nm (25nm)	590 nm (20nm)	75	5 µM	TE buffer

The data at 60 min of inhibition for each concentration of inhibitor was used to determine IC_{50} values of a CYP1A1 inhibitor, using GraphPad Prism.

Reagents and Media

- Tris-HCl
- EDTA
- 7-Ethoxyresorufin (7-ER).

TE Buffer (50 ml)

500 µl of 1 M Tris-HCl pH 7.4 was added to 100 µl of 0.5 M EDTA pH 7.5 and was made up to a volume of 100 ml with pure water.

2.10 Western blot of CYP proteins expressed in yeast

2.10.1 Protein quantification in yeast

Recombinant yeast cells, containing *CYP* genes, were grown in 3-4 ml of YPD complete medium, without any selection, overnight (~18 h) at 30°C by shaking at 200 rpm. 5X 10^6 cells were usually spun down at 13,000 rpm for 1 min to remove supernatant which was essentially the exhausted YPD medium. The cell pellet was washed 3X in 1 ml 1X TE buffer. The cell pellet was re-suspended by vortexing in 200 µl of cell lysis buffer (5.7 µl 10X SDS, 1 ml 10X TE buffer and made up to 10 ml with deionized pure water).

The suspension was sonicated (Grant Ultrasonic bath XUBA1) twice for 10 sec, to enable efficient release of proteins by the yeast, *Saccharomyces cerevisiae*. The sonicated cell suspension was incubated in 99°C for 10 min to open the yeast membrane.

The suspension was centrifuged in a centrifuge, pre-cooled to 4°C, at 16000X g for 20 min and immediately was placed on ice gently. The supernatant was transferred to a new Eppendorf tube.

The proteins in the lysed cells were diluted 1:20 (to each 5 µl cell lysate was added 95 µl 1X TE buffer). This allowed calculation of total protein concentration in each sample of cell lysate. All protein samples obtained from cell lysis were stored at -20°C when not in use. Protein degradation was avoided by minimizing freeze-thaw cycles.

2.10.2 Preparation of protein sample for Western blots

Proteins from 2X 10^6 cells (i.e. 20-30 µg of protein), after cell lysis, contained in 20 µl was added to 20 µl of 5X loading sample buffer. The mixture was heated to 95°C for 5 min, for denaturation of proteins, and then centrifuged at 16000X g for 1 min. The Eppendorf tubes containing denatured proteins were returned on ice and the contents were centrifuged for 15 min at maximum speed. 20 µl of this mixture was loaded on to wells of an SDS-polyacrylamide gel. Standard protein ladder (5 µl; Bio-Rad Catalogue No 1610398) was loaded on one of the extreme left or right hand side well of the gel.

2.10.3 Buffers used for Western blotting

10X TBS: 87.6 g of NaCl + 60.5 g Tris Base + 800 ml water to make up to 1 L, pH adjusted to 7.4 by adding around 10 ml 1M HCl.

1X TBST: (50 mM Tris-Cl, pH 7.4; 150 mM NaCl). 8.76 g of NaCl + 6.05 g Tris Base + 800 water to make up to 1 L adjusted to pH 7.4 by adding around 10 ml 1M HCl; 1 ml Tween 20 was added slowly until it is a homogenous solution was made.

2.10.4 Gel Castings

Table 2.8. Polyacrylamide gel for resolution of proteins in cell lysate, via Western blotting; volumes of reagents required for 10 ml solutions are shown.

Water	4.7 ml
1.5M Tris-HCl(p H 8.8)	2.5 ml
30%Acrylamide mix	2.5 ml
10% SDS	100 µl
10% Fresh Ammonium persulfate	100 µl
TEMED (*N*,*N*,*N*,*N* -tetramethylethylenediamine	10 µl

Table 2.9. Stacking gel for Western blotting.

Water	3.1ml
0.5M Tris-HCl (pH 6.8)	1.25ml
30% Accrylamide mix	600µl
10% SDS	500µl
10% Fresh Ammonium persulfate	50µl
TEMED (*N*,*N*,*N*,*N* -tetramethylethylenediamine	10µl

2.10.5 Introduction to Western Blotting

Western blotting or immunoblotting allows investigators to determine, with a specific primary antibody, if a protein antigen at all exists in a particular cell environment and, if it did, at what relative levels. Often, the relative amounts of a protein in different samples are gauged to a rough approximation using Western blots.

Briefly, 1) The sample is prepared from microsomes or yeast cells that are homogenized in a buffer that protects the protein of interest from degradation; 2) The sample is separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a synthetic membrane for detection; 3) The membrane is incubated with generic protein (such as milk proteins or bovine serum albumin) to bind to any remaining unoccupied places on the membrane. A primary antibody is then added to the solution which is able to bind to the specific protein; 4) A secondary antibody-enzyme conjugate, which recognizes the primary antibody is added to illuminate the location where the primary antibody was bound.

2.10.6 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

This technique was based on a modified version of that described by Laemmli (Laemmli, 1970). The Mini-PROTEAN 3 Electrophoresis system purchased from Bio-Rad was used in my studies. 10% separating gels consisted of final concentration of 7.5% acrylamide/bis-acrylamide (30%) (Sigma-Aldrich, Cat No A6050), 1.5 M Tris-HCl pH 8.8, 0.1% SDS, 0.001% N-,N'-Tetramethylethylenediamine (TEMED) (Sigma-Aldrich, Cat No T7024) and 0.001% ammonium persulphate (APS) (Sigma-Aldrich, Cat No

A3678), and were poured into the casting stand units to within 0.5-1.0 cm from the bottom edge of the well comb and the gels were left to polymerise for about an hour. The stacking component of the gel was made immediately prior to electrophoresis. The stacking gel consists of 4% acrylamide/bis-acrylamide (30%), 0.5 M Tris-HCl pH 6.8, 10% SDS, 10% TEMED and 10% APS and was poured over the separating gel 0.25 cm from the top of the gel kit, the well comb was then inserted before the gel sets. Polymerisation of the gel takes about an hour. The completed gel running apparatus was then placed into its gel tank and the upper and lower reservoirs were filled with appropriate amounts of running buffer which consisted of 25 mM Tris-HCl pH 8.6, 192 mM glycine and 0.1 SDS (w/v). Samples for electrophoresis were prepared at required dilutions using the sample buffer consisting of 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% Bromophenol blue and 0.125 M Tris-HCl pH 6.8. The diluted samples were then boiled for 2-3 min to denature the proteins and then cooled on ice. Up to 20 µl of boiled samples were loaded on each well. 5-10 µl Bio-Rad standards (Bio-Rad, Cat No 161-0309) were loaded usually on one side of the SDS-polyacrylamide gel. Electrophoresis was performed using constant current, using a power supply (Bio-Rad, Cat No 164-5050). For stacking the proteins, the gels were run at 8 mA per gel until the loading dye had concentrated at the top of the separating gel. The current was then increased to 16 mA per gel until the blue running dye had reached the bottom of the gel or the marker had reached a suitable resolving distance.

2.10.7 Western blotting

Once the proteins were separated using SDS-PAGE on a 10% SDS-polyacrylamide gel as described above, the gel was carefully taken off from the tank. The stacking gel was scraped away from the separating gel and discarded. The separating gel was then soaked in Cathode buffer (25 mM Tris, 20% methanol, 40 mM 6-aminohexanoic acid), and placed onto 3X Whatman chromatography paper (Whatman, Cat No 3030335) pre-soaked with Cathode Buffer on blotter. A piece of nitrocellulose membrane (Millipore, Cat No VX 466008), roughly the size of the gel, was soaked in Anode buffer II (25 mM Tris, 20% methanol, pH 10.4) for 2-3 min, and then layered over the separating gel, ensuring that no air bubbles were trapped between the gel and the nitrocellulose sheet. Three more pre-soaked Whatman papers in Anode buffer II were placed over the top of the nitrocellulose sheet, and three Whatman papers soaked in Anode buffer I (300 mM Tris, 20% methanol, pH 10.4) were placed on top of previous sheet of Whatman paper. The blotter cover was gently placed and the blotter was run at a constant current of 0.8 mA/ cm^2 for 2-3 h. Once transfer was completed, the nitrocellulose sheet was placed in a plastic tray containing 50 ml of PBS/ 0.1% Tween (polyoxyethylene-sorbitan monolaurate) for 10 min with gently shaking, and PBS/ 0.1 Tween solution was poured off, 5 ml blocking solution (10% fat free milk (Bio-Rad, Cat. No. 170-6404) in PBS) was added, and incubated for 1 h with gently shaking. The nitrocellulose sheet was rinsed briefly with PBS/0.2% Tween and washed once in PBS/0.2% Tween for 15 min followed by three 5 min washings in PBS/0.2% Tween. The blot was then incubated in 25 ml of antibody solution that contained the primary antibody and incubated at 4ºC overnight with gentle shaking. The antibody was diluted in 1% milk, PBS/0.2% Tween solution at least at 1:1000 dilution. The blot was then washed three times with PBS/0.2% Tween each

time for a period of 10 min. After washing, the blot was incubated in 30 ml of secondary antibody solution that contained secondary antibody at 1:3000 dilution in 1% milk, PBS/0.2% Tween for 2 h at room temperature with gentle shaking. The secondary antibody was discarded and the blot was washed twice in PBS/ 0.2% Tween followed by washing twice with PBS. Finally, the blot was rinsed twice with sterile water. The blot was placed on the reagent bath containing 5 ml of enhanced chemiluminescence Western blotting detection reagent solution A and solution B (Santa Cruz Biotechnology, Cat No SC-2048), mixed by inversion, the water was poured off from the blot and the mixed Luminol reagent solution was added to the membrane with protein side facing up for 2 min at room temperature. The excess Luminol reagent was poured off and the membrane was lifted with a pair of forceps to drain excess reagent. The membrane was tightly covered, protein side facing up, with a single layer of plastic wrap. The chemiluminescence was detected with the Gel Doc™ system (Bio-Rad) with multiple exposures of 15 seconds, up to a period of 10 min.

2.11 Biotransformation with CYP-expressing recombinant yeast cells under the control of the ADH2 promoter

2.11.1 A Typical Biotransformation Experiment

(1) Pre-culture-1: A scoopful of CYP-containing 'stable' yeast cells were inoculated, from a fresh YPD (complete medium) plate, into 50 ml 1X YPD in a 500 ml conical baffled flask; shaken at 30°C, 220 rpm, ~17 h; baffled flasks were used for vigorous aeration; after all a CYP enzyme would have to utilize oxygen to metabolize the starting material.

(2) Pre-culture-2: Pre-culture- cells were harvested and re-suspended in 50 ml 1X YPD in a 500-ml conical baffled flask; shaken at 30°C, 220 rpm, 14 h;

(3) Pre-culture-3: Pre-culture- cells was harvested from and re-suspended in 50 ml 1X YPD in a 500 ml conical baffled flask; shake at 30°C, 220 rpm, 14 h;

A gradual increase of OD_{600} (optical density measured at 600 nm) was observed with each day of culture and this also translated to an increase of CYP activity (using pro-fluorescent substrate), from pre-culture 1 to pre-culture 3.

(4) Main culture (i.e. the cells that are involved in biotransformation).

a. Cells harvested from Pre-culture-3, were re-suspended (~100 OD_{600} of cells) in 5 ml 1X SD (minimal medium) in four 50 ml baffled flasks;

b. Codeine was added to cultures (final concentration, 50 µM) from a 50 mM stock solution in 100% DMSO; the final concentration of DMSO in the cultures was < 0

c. Flasks were shaken at 30°C, 220 rpm, 24, 48, 72, 96 h;

d. Cells were harvested every 24 h for assessing biotransformation (i.e. amounts of bio-transformed products).

e. For the 48 h, 72 h, 96 h cultures, 0.2 ml of 50% glucose was added every 24 h of growth, to replenish levels of glucose to 2%.

Note: Glucose is exhausted after 12 h of growth. Hence, every other 12 h, cells make the CYP enzyme. The *ADH2* promoter (ADH2p) is repressed in the presence of glucose and

induced in the presence of ethanol. As glucose is converted to ethanol during yeast cell division (i.e. yeast cell growth), the ADH2p is induced (i.e. when CYP proteins are made). Therefore, over a 96 h time period, 48 h will be spent by the cells on growth (cell division) and the other 48 h on CYP production. During the time CYP enzyme is produced, (codeine to morphine) biotransformation will take place.

2.11.2 Composition of YPD and SD Medium for growth of cells for biotransformation

YPD = 5 g of Yeast extract(LP0021, OXOID)+10 g Proteosepeptone (LP0085 OXOID) +10 g Glucose add up 500 ml pure water and autoclave

SD-agar = 3.35 g yeast nitrogen base(BD291940, DIFCO) 10 g glucose 10 g agar 1 pellet of NaOH add up to 500 ml pure water

SD-MM = 3.35 g yeast Nitrogen base (291940, BD DIFCO) + 480 ml pure water+ 20 ml 50%.

2.11.3 Thin Layer Chromatography Procedures

1) The cells were spun down from each flask for 5 min at 13,000 rpm.

2) Transferred the total amount of the recovered supernatant into separation funnel with a glass stopper.

3) 200 µl of 0.1M ammonia was gently added.

4) Butanol was used to extract codeine/morphine; extracted 3X with 5 ml pf 1-butanol. I made sure the layers were well separated before removing the aqueous layer.

5) The organic layer was passed through 0.2 µm Ministart high-flow syringe filter to remove cell debris; alternatively, paper filters could be used.

6) Eluent was transferred from the filter into the Büchi evaporator flask and evaporated at 65°C.

7) The extracted residue was re-suspended in 5 ml of methanol.

A laboratory set up for Codeine/Morphine extraction.

2.11.4 TLC Buffer

For detection of codeine and morphine, the solvent systems that were attempted were:

1) Dichloromethane (DCM) : Methanol : Ammonia = **24 : 2.8: 0.4**
2) Ethyl acetate : Methanol : Ammonia = **85 : 10 : 5**
3) Toluene : Acetone : Ethanol : Ammonia = **40 : 40 : 6 : 2**

Solvent system **(3)** provided the best separation.

All solvents were HPLC grade.

- Dichloromethane (DCM), Sigma Cat No 1.13713,
- Methanol solution contains 0.1 %(v/v) formic acid, Sigma Cat No 632546,
- Chloroform for HPLC 0.5-1% ethanol as stabilizer, Sigma Cat No 439142,
- Toluene, Sigma Cat No 648566,
- Acetone, Sigma Cat No 439126,
- Water with formic Acid Hypergrade for LC-MS Sigma Cat No 1.59013,
- Ethyl acetate Sigma Cat No 1.03649,
- 1-Butanol for liquid chromatography Cat No 1.01988,
- Ammonia anhydrous Sigma Aldrich Cat No 7664-417.

Chapter 3 Cloning and construction of yeast plasmids for expression of 17 human *CYP* genes (most of which used for Drug Metabolism studies), synthesized using yeast biased codons

3.1 Introduction

Cytochrome P450 (CYP) enzymes belong to a large superfamily of proteins that exist in all eukaryotic and some prokaryotic organisms. The first heterologous expression of a functional human CYP was demonstrated in the baker's yeast, Saccharomyces cerevisiae (Ton et al., 2004). Yeast was preferred as a heterologous system for expression of eukaryotic CYP450 proteins because of

(1) Yeast cells' high efficiency and easy mode of growth which is economically much cheaper compared to other higher eukaryotic cells,

(2) Vector availability with strong constitutive or inducible promoters (*GAPDH* or *GAL* promoters) that would allow proficient gene transcription of heterologous genes, such as *CYP450* genes, during cell division (Qin et al., 2011),

(3) Availability of robust host strains that can overexpress membrane integrated proteins, such as cytochrome P450 reductase (CPR) which is absolutely essential for CYP enzyme activity (Kandel et al., 2014), and most importantly the

(4) Presence of endoplasmic reticular (ER) membranes in yeast, similar to that in human cells, which normally house most eukaryotic CYPs enzymes; a few human CYPs, however, are associated with mitochondrial membranes which also play similar roles in both human and yeast cells (Schuck et al., 2009).

Although these traits underline yeast's advantages compared to other eukaryotic hosts, the level of expression of most human CYP enzymes in yeast is low and most of the proteins that are produced have low enzymatic activity (Walmsley et al., 2002). In this thesis, attempts have been made to overcome these flaws so that yeast systems that produce CYP enzymes could be made available for wider commercial use. The main aim was to address the persistent problems of low expression and activity of human CYP enzymes produced in yeast.

Codon optimized genes have commonly been used to enhance heterologous gene expression of proteins specifically in the context of synthetic biology, where cells are manipulated for optimal synthesis of chemicals and their metabolites. The strategy used in the codon optimization process involves replacement of rare codons with more frequently occurring ones in highly expressed genes that code for native proteins of host cells. It is well documented that production of functional heterologous proteins in a host organism is enhanced with the use of host-biased codons and that this influences protein yields (Milda and Alma, 2012). The questions that come to mind are:

(1) Why do different organisms prefer different codons?

(2) How does one envision codon bias?

(3) How does codon bias affect protein expression? and

(4) Does codon bias somehow modify the host to improve protein expression?

There are 61 nucleotide triplets that code for 20 essential amino acids that are used by most organisms to synthesise proteins. Besides the 61 triplets, there are 3 nonsense codons, also known as stop codons where transcription stops during gene transcription.

In the ribosome, the codons are read by complementary tRNA coupled with the charged amino acids. How the codons are used and their frequency of use differs from one species to another and also even between proteins, expressed at low or high levels, within the same organism. In the past, it was strongly believed that evolutionary forces have evolved in the usage of certain codons by an organism (which is also referred to as selective equilibria between codons of the same species; Sharp et al., 2010).

The adaptation index in the expression of a foreign protein in a host organism is identified with the help of correlation between codon bias of the expressed gene and its expression levels (Sharp et al., 1987; Chung et al., 2012). The index predicts the expression levels of endogenous genes present in the genome to which they belong but it does not measure the nature of adaptation or preference and this makes it more difficult to assess the likely compatibility between a gene that is expressed and the host candidate. To establish the codon preference, some major components can be used to compress the multi-dimensional information into two-dimensional maps. This enables a convenient way to see the dissimilarities in the codon preferences between organisms. Therefore, this study is based on the strategy to improve expression of foreign genes by altering their rare codons so that they will be more reflecting the codons of baker's yeast without any changes in the amino acid sequence of the encoded protein.

It is likely that codon usage and iso-acceptor tRNA concentrations have co-evolved suggesting that the selection for this co-evolvement gives rise to a greater chance of increased induction of transcription of genes which can lead to higher expression of proteins (M. Bulmer, 1987). It was believed that the more the codons of rarely used codons appear in a gene the higher the possibility that the heterologous protein will have

a low expression level (Del Tito et al., 1995). It was also reported, in a similar study, that two to five of the codons from CGG, AUA, CUA, CGA and CCC that may occur in tandem, and in clusters, have significant up-regulatory effects on expression of test proteins (Rosenberg et al., 1993).

Yeast biased codons may not be the only factor which contributes to the increased levels of expression of foreign genes in yeast. The particular yeast strain which acts as a host may play a very huge role in achieving high expression levels together with the transcriptional promoter which lies upstream of the gene to be expressed (X. Wu 2004). If there is efficient translation, it is absolutely imperative that the folding of the polypeptide chain occurs correctly for the protein to manifest appropriate activity.

3.2 Outline of Chapter 3

The goals of this chapter were to:

(1) Construct episomal 2µ (multi-copy) plasmids for expression of human CYP proteins in yeast, using *CYP* genes that were chemically 'synthesised' with yeast biased codons,

(2) Express 'synthesised' human *CYP* genes using yeast biased codons (referred to as *CYP_yc*), as CYP proteins within yeast cells using episomal plasmids,

(3) Express 'native' human CYP genes which were earlier isolated from a human liver cDNA library (*CYP*) within yeast cells using episomal plasmids,

(4) Compare expression of activities of a specific CYP enzyme produced from 'synthesised' *CYP_yc* gene, as in (2), with CYP enzyme produced from 'native' *CYP_na* gene, as in (3).

Results from experiments (1) to (4) would answer the question, do 'synthesised' genes produce active enzymes that are as good as (or, better than) the enzymes produced from 'native' genes? The results, presented in this Chapter, show that the enzymes produced from 'synthesised' human *CYP* genes are more active than the ones produced from the 'native' genes. Most likely, this is because a *CYP_yc* gene produces more CYP protein than a *CYP_na* gene, per unit number of cells. The methodologies, used in this Chapter, were earlier described in Chapter 2 (Material and Methods). The protocols in Chapter 2 were followed to clone and express a specific *CYP_yc* gene, chemically synthesised with yeast biased codons.

3.3 Construction of recombinant DNA molecules

One of the main aims of the project was to devise new systems for expression of functionally active human CYP enzymes in baker's yeast.

Within this aim, the first goal was to find out how a 'chemically synthesized' human *CYP* gene, with codons that are yeast biased (_yc), fare against a *CYP* cDNA ('native'; _na) isolated from a human liver cDNA library in terms of (a) 'CYP enzyme activity' , (b) 'CYP yield' and (c) 'co-expression with cytochrome b5 for some CYPs'. It has been reported that co-expression of the cytochrome b5 protein with a CYP and a cytochrome P450 reductase (CPR) provides higher enzyme activity to certain CYPs. A CPR is essential for the activity of all CYPs (Kendel et al., 2014).

For the above comparative analyses, 17 human *CYP* genes were chosen. Most of the 17 genes are used for Drug Metabolism studies, others having crucial roles in the synthesis of steroids and fatty acids that are endogenous to human cells. All the genes were synthesized using yeast biased codons and were first cloned into the commercially available basic vector, pUC57 (Thermo Fisher, UK).

The pUC57 plasmids, bearing 'synthetic' *CYP* genes, were amplified by transforming into bacterial DH5α cells. All seventeen *CYP* genes were isolated from the pUC57 plasmids using restriction enzymes that cut at specific recognition sites at the 5' and 3' end of the genes. The *CYP* genes were then ligated into a shuttle vector of choice (i.e. pSYE263) which had been constructed in-house previously. The resultant pSYE263 derived plasmids were amplified in bacterial cells and were then transformed into yeast. During yeast cell division, the transformants replicate extra-chromosomally within yeast cells. For maintenance of plasmid, the yeast cells must be grown in selective synthetic defined (SD) medium. Extra-chromosomal plasmids cannot be maintained in complete YPD (yeast + peptone + dextrose) medium. A plasmid is gradually lost after 10 generations of growth, each yeast generation being of 90 min duration.

3.3.1 Yeast transformation

The yeast transformation protocol, used in this Chapter, has been described in Chapter 2, Section 2.5.4.

The yeast strains that were used were:

(1) YAB79 that contains *ΔhRDM* and cytochrome *b5* genes at the *LEU2* and *TRP1* loci; and

(2) YY7 that contains only *ΔhRDM* at the *LEU2* locus).

The constructions of these strains are described in more detail in Chapter 4, Section 4.4.4.

3.3.2 Plasmid vectors

The yeast extra-chromosomal vector used, in the construction of recombinant DNA plasmids for expression studies in this project, was the episomal 2μ-based vector pSYE263 (Figure 3.1). It contains the yeast *ADH2* promoter which belongs to one of the two alcohol dehydrogenase genes of yeast. Alcohol dehydrogenases are responsible for oxidation of ethanol to enable its removal from living cells.

The vector pSYE263 was already available in the lab and a small aliquot (10μl) of a bacterial glycerol stock from -80°C was inoculated into LB broth, grown overnight at 37°C and the vector plasmid was isolated. The 573 bp *ADH2* promoter fragment in the plasmid pBluKS(+)/ADH2p (Figure 3.2) was used to construct the episomal plasmid pSYE263, containing the *ADH2* promoter. In order to isolate the 573 bp fragment from the plasmid, it was first extracted from bacterial cells which were grown from a glycerol stock kept at minus 80°C.

Both the plasmids, pSYE263 and pBluKS(+)/ADH2p, bear a β-lactamase gene and a range of restriction sites, as depicted in Figures 3.1 and 3.2.

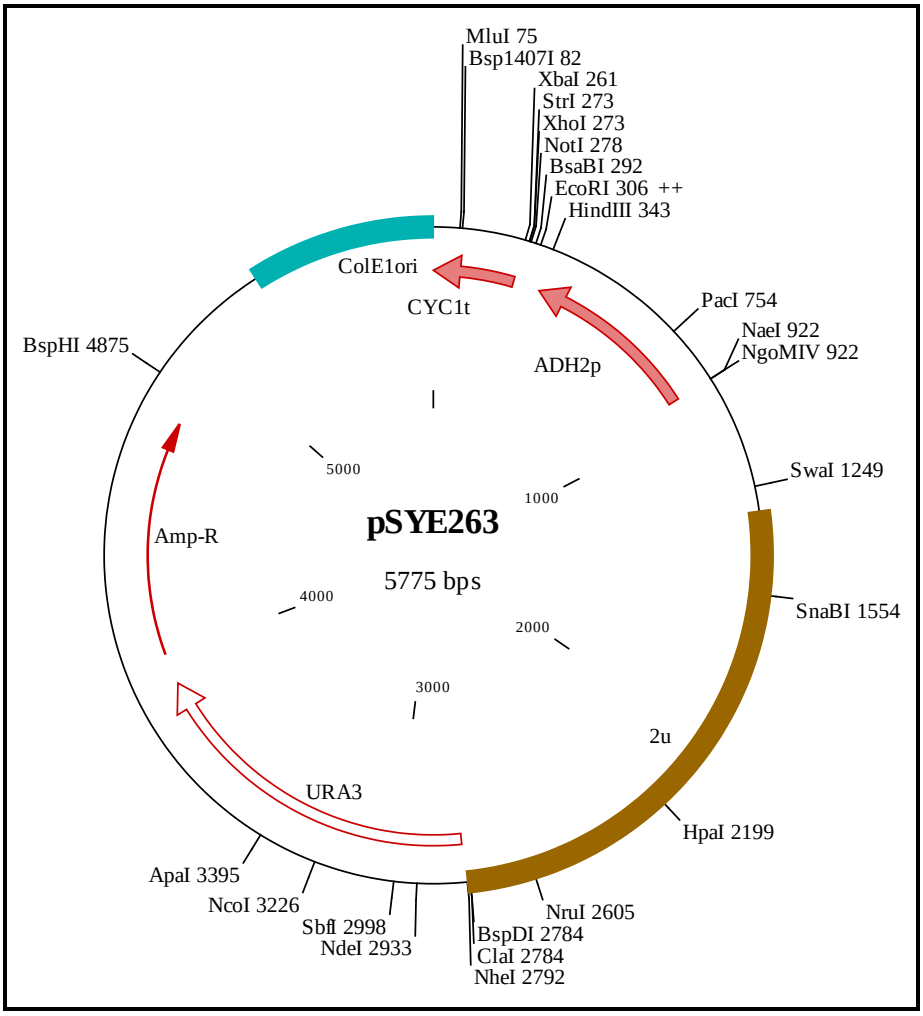


Figure 3.1. The plasmid map of pSYE263, the 2µ-based episomal plasmid containing the yeast *ADH2* promoter which was isolated from the plasmid pBluKS(+)/ADH2p (Figure 3.1). The restriction sites shown on the map occur in the plasmid only once.

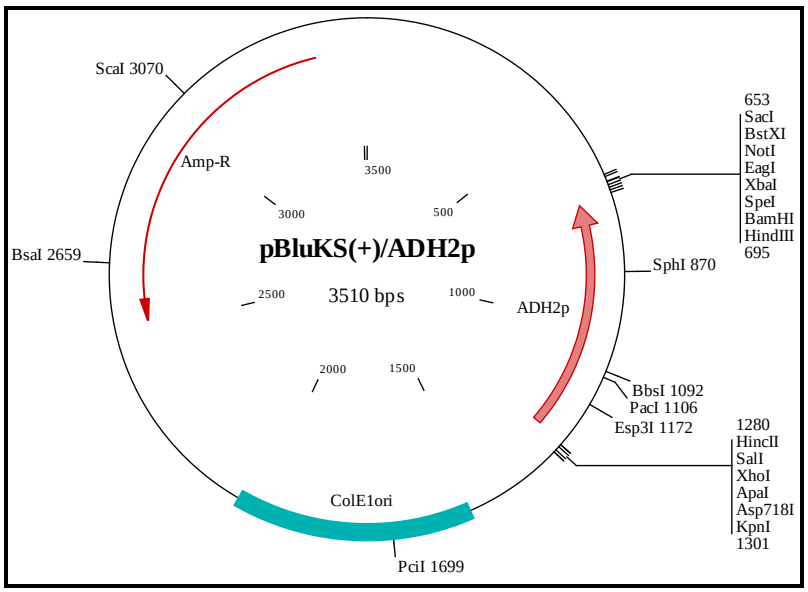


Figure 3.2. Shows the map of the basic plasmid that contains the 573bp-*ADH2* promoter. This was isolated to create the plasmid pSYE263 (Figure 3.1). The restriction sites shown on the map occur in the plasmid only once.

3.4 Cloning of *CYP3A4_yc* gene in the episomal plasmid pSY263 for expression of human CYP3A4 enzyme

3.4.1 Cloning of *CYP3A4_yc* gene in the episomal plasmid pSY263

CYP3A4 is one of the ten to twelve CYP enzymes which are widely used for Drug Metabolism studies during the process of drug development (Zanger et al., 2013). At first, the plasmid pUC57/h_CYP34_yc (Figure 3.3) was digested with the restriction enzymes *BamH*I and *Xba*I. The 1524 bp insert fragment containing the human *CYP3A4_yc* gene was isolated from an agarose gel and the 2700 bp pUC57 vector fragment was ignored (Figure 3.4). The *CYP3A4_yc* gene insert was then cloned in pSYE263, which had been

digested also with *BamH*I and *Xba*I restriction enzymes (Figure 3.5) to obtain the plasmid pSYE263/h_CYP3A4_yc (Figure 3.6).

At this point one should emphasise that all *CYP_yc* genes, containing yeast biased codons, were constructed in-house (by Professor Chaudhuri, my supervisor) but were chemically synthesised by GeneWiz (USA). After receipt of the synthetic genes, all further DNA manipulations were performed by me during the course of the work described in this thesis.

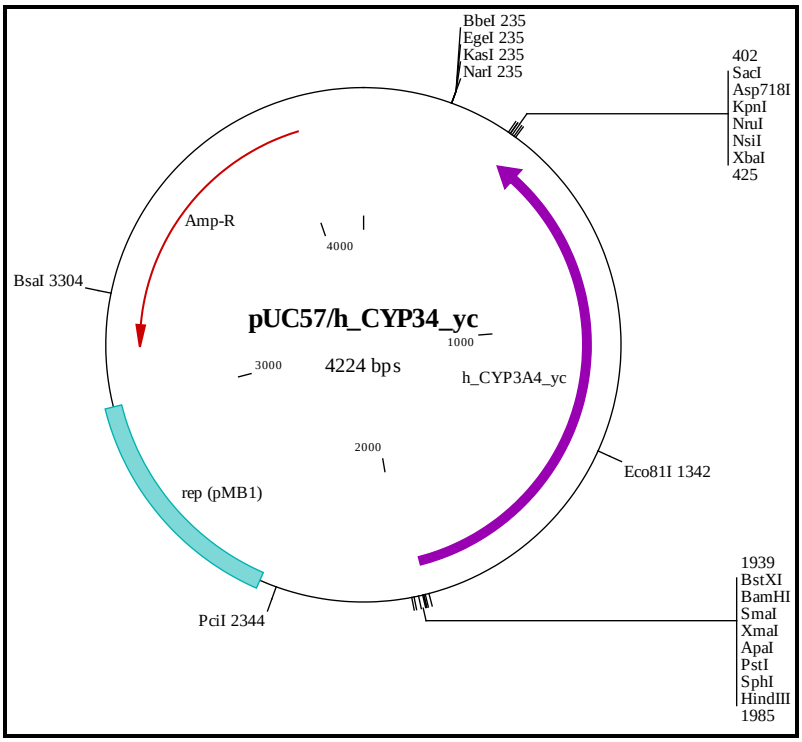


Figure 3.3. The plasmid map for pUC57/h_CYP34_yc, which contains the human *CYP3A4* gene synthesised using yeast biased codons (*h_CYP34_yc*), cloned at the BamHI and XbaI sites of the plasmid. The BamHI-XbaI *h_CYP34_yc* insert fragment was isolated for further cloning in the episomal vector pSYE263 and integrative vectors described in Chapter 4. The restriction sites shown on the map occur in the plasmid only once.

Similar pUC57 based plasmids were constructed for the rest of the *CYP* genes described in this Chapter (Chapter 3).

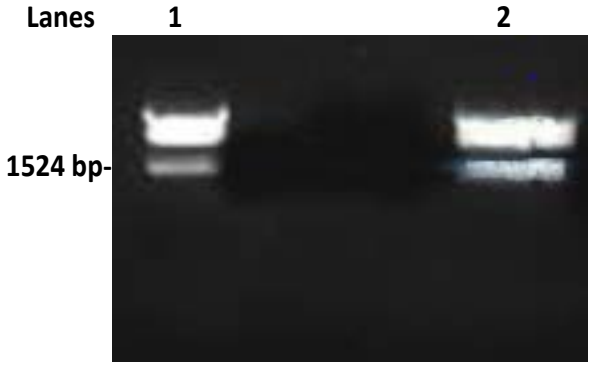


Figure 3.4. It shows the agarose gel that has fractionated the DNA fragments obtained after digestion of the plasmid pUC57/h_CYP34_yc after digestion with restriction enzymes, BamHI and XbaI.

The 1524bp *h_CYP34_yc* gene fragment (Figure 3.4) was isolated from the gel and cloned in the yeast expression vector pSYE263, a 2u-based episomal plasmid that contains the *ADH2* promoter and the *CYC1* transcription terminator. In between the promoter and the terminator are the multi-cloning sites in which the gene *h_CYP34_yc* was cloned through DNA ligation. In order to clone the gene, pSYE263 was first digested with *BamH*I and *Xba*I and the vector fragment was isolated (Figure 3.5).

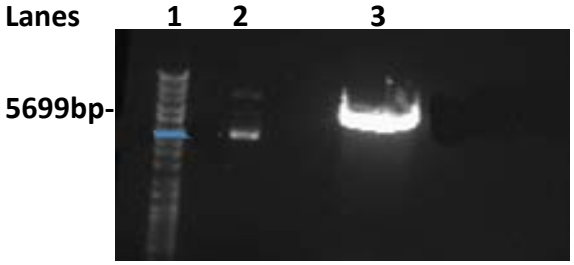


Figure 3.5. The *BamH*I-*Xba*I digested pSYE263 linearised vector fragment (5699 bp) was isolated from the agarose gel. Lane 1, DNA ladder of established DNA fragments (commercially available; New England Biolabs). Lane 2, uncut vector; lane 3, digested vector which was isolated from the gel for ligation with the insert (Figure 3.4) obtain the plasmid pSYE263/h_CYP3A4_yc.

The 5699 bp pSYE263 vector fragment and the 1524 bp *h_CYP34_yc* insert fragment were ligated with the help of the enzyme DNA ligase. After ligation and transformation in *E. coli* DH5α cells, individual colonies were grown for preparation of DNA using the

alkaline lysis method (Chapter 2, Section 2.4.1.8). The resultant plasmid DNA was named pSYE263/h_CYP3A4_yc (Figure 3.6). The authenticity of the plasmid was confirmed by multiple restriction enzyme digests. One such digest with the enzyme *Nde*I is shown in Figure 3.7.

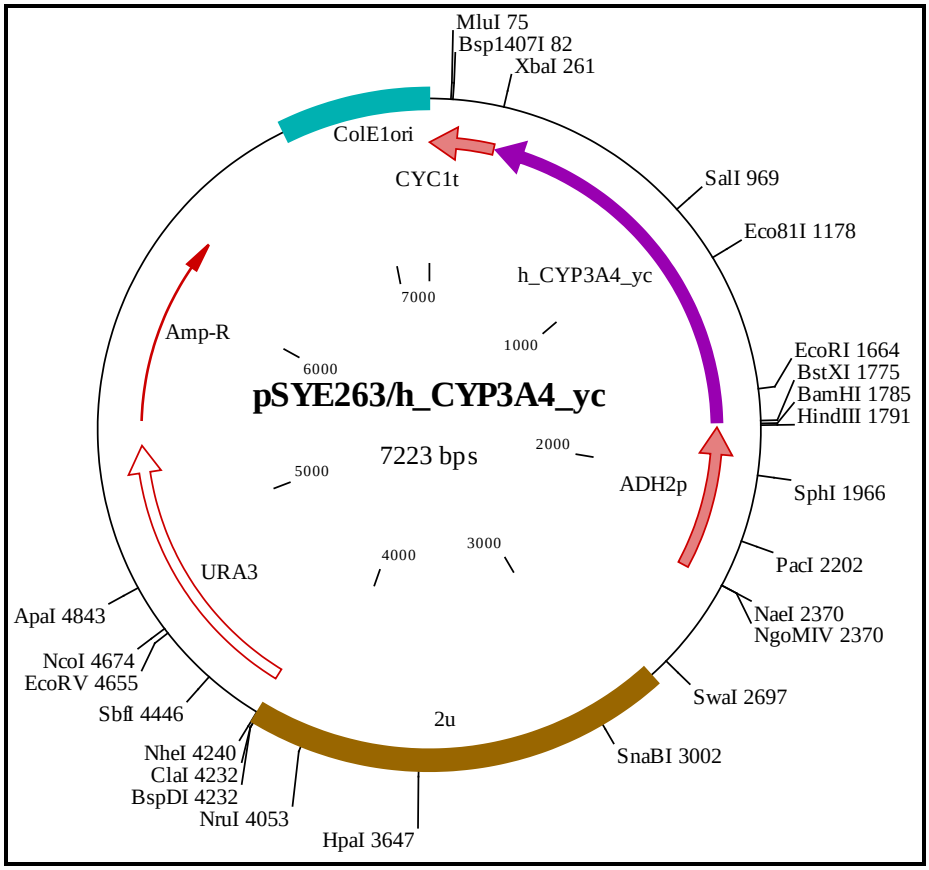


Figure 3.6. It shows the restriction enzyme map of the episomal, 2µ-plasmid pSYE263/h_CYP3A4_yc that was used for expression of the *h_CYP3A4_yc* gene, to produce active human CYP3A4 enzyme. The restriction sites that are shown on the map occur in the plasmid only once.

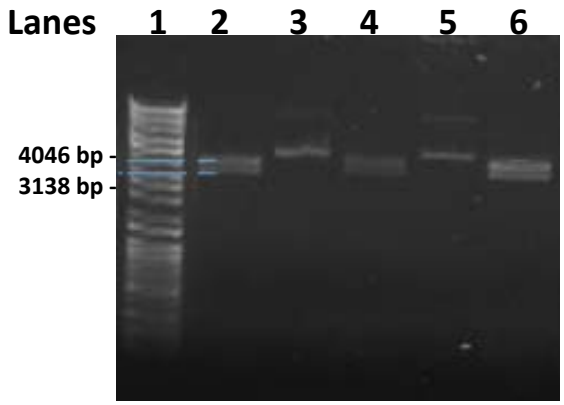


Figure 3.7. The agarose gel that shows the expected DNA fragments when the plasmid pSYE263/h_CYP3A4_yc was digested with the restriction enzyme *Nde*I (lanes 2, 4, 6). Lanes 3, 5 show the uncut plasmid. Lane 1, DNA ladder of established DNA fragments (commercially available; New England Biolabs).

3.4.2 Comparison of CYP3A4 enzyme activities expressed by 'synthesised' *CYP3A4_yc* and 'native' *CYP3A4_na* genes, both encoded by the episomal plasmid pSY263, using a fluorescence-based assay

The plasmid pSYE263/h_CYP3A4_yc (Figure 3.6) and pSYE263/h_CYP3A4_na (containing the native *CYP3A4* gene; plasmid map not shown; published in an earlier thesis from this laboratory) were transformed in the yeast strain YAB79 that contains *ΔhRDM* and cytochrome *b5* genes at the *LEU2* and *TRP1* loci. The gene *h_CYP3A4_na* represents the human *CYP3A4* gene isolated from a human liver cDNA library. The transformants obtained after transformation of the two plasmids in the yeast strain YAB79 were designated as:

(a) Episomal 3A4_yc, and

(b) Episomal 3A4_na.

Transformants from the empty plasmid pSYE263, which does not encode any foreign *CYP* gene, were used as a control for comparison of human CYP3A4 enzyme activities from the *CYP3A4_yc* and *h_CYP3A4_na* genes (Figure 3.8).

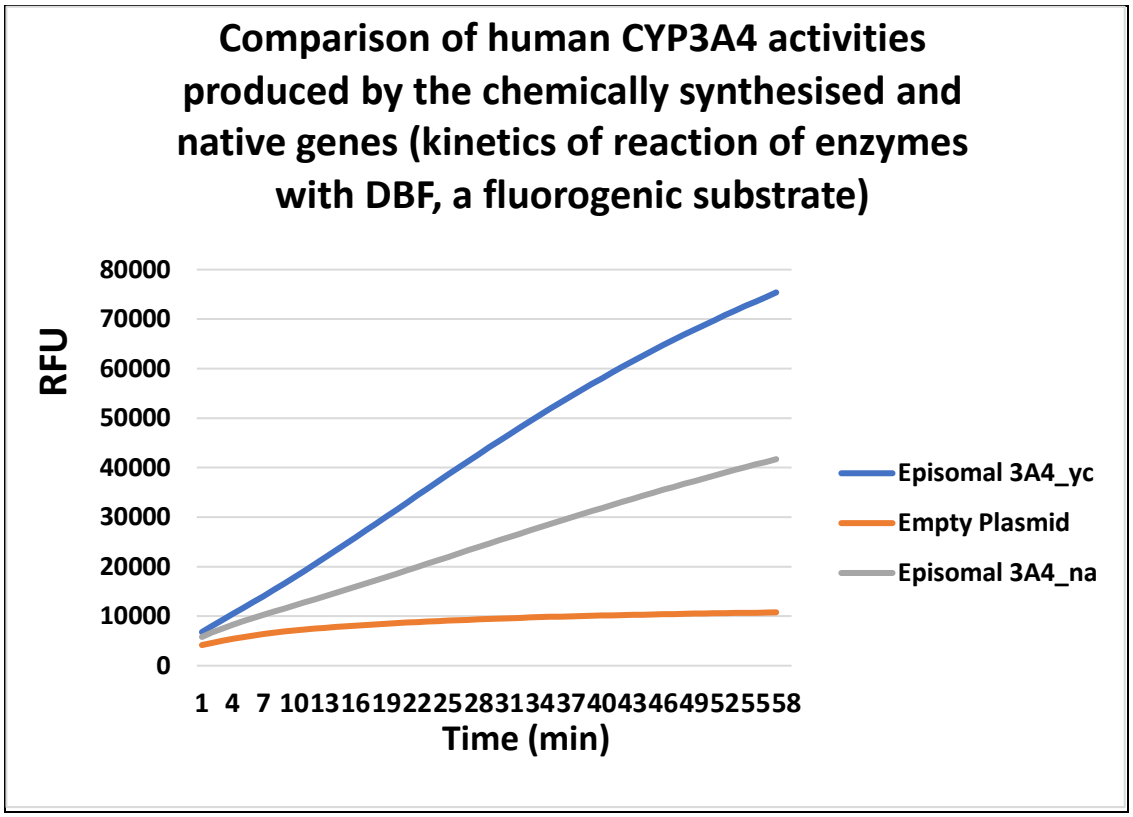


Figure 3.8. The graph shows the kinetics of activity (i.e. enzyme activity with progression of time) of the CYP3A4 enzyme which was expressed in yeast from (a) the 'synthesised' *CYP3A4_yc* gene (blue) and (b) the 'native' *CYP3A4* gene (grey) using the same number of cells (1X 10^7); both genes were co-expressed with the modified human CPR gene, ΔhRDM, and cytochrome b5. Rate was measured in terms of relative fluorescence units (RFUs), using dibenzylfluorescein (DBF) as substrate. DBF is de-alkylated by CYP3A4 to form the fluorescent product flourscein. The empty plasmid (pSYE263 containing no CYP3A4 gene), when expressed in yeast, showed no activity, as expected (beige).

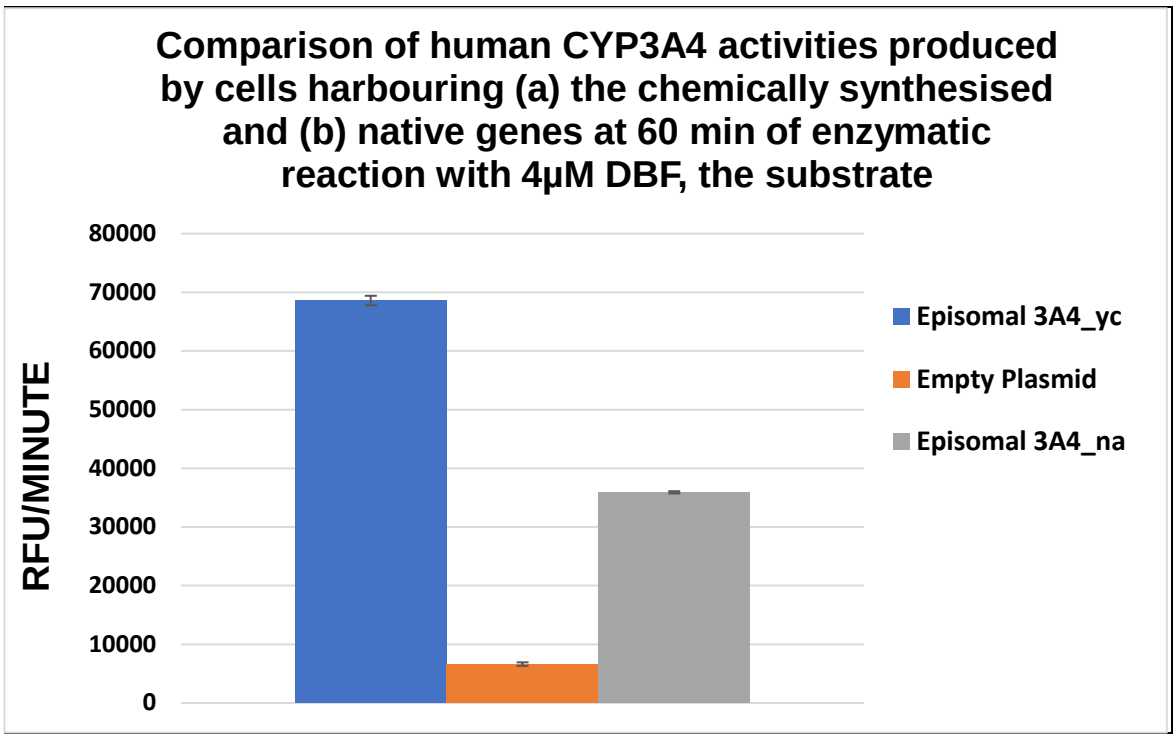


Figure 3.9. The bar chart shows a comparison of human CYP3A4 enzyme activity obtained from same number of yeast cells (1X 10^7), expressing 'synthesized' *CYP3A4_yc* and *CYP3A4_na* genes, obtained at 60 min of reaction of cells with DBF, the fluorogenic substrate. The data represent mean ± S.D. of three independent experiments.

The chart in Figure 3.9 shows the relative CYP3A4 activity of three different cell types: cells which contain (a) an episomal plasmid bearing the *CYP3A4_yc* gene, (b) an episomal plasmid bearing the *CYP3A4_na* gene, and (c) an empty episomal plasmid. The cells that do not express any *CYP3A4* gene show basal residual fluorescence from the substrate DBF (beige bar).

The results from Figures 3.8 and 3.9 would indicate that there is more protein expressed from the 'synthesized' *CYP3A4_yc* gene than from the 'native' *CYP3A4_na* gene because, firstly, both genes would produce exactly the same enzyme and therefore the protein produced from the two genes should have exactly the same activity. However, as

would be expected, the gene with the yeast biased codons should provide a more stable mRNA (i.e. more mRNA would be transcribed relative to what would be transcribed from the 'native' gene). More mRNA would translate to more protein, relative to what would be produced by the 'native' mRNA. This would have need to be confirmed by performing Western blot analysis on equal number of cells that express the two variants ('synthesized' and 'native') of the *CYP3A4* gene (Figure 3.10).

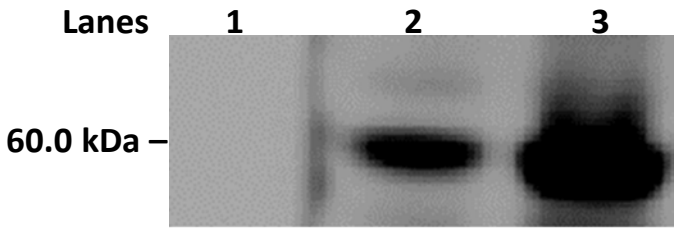


Figure 3.10. Western blot showing the amounts of CYP3A4 protein being expressed by equal number of cells (1X 10^6). The total cellular protein obtained from these cells was loaded on wells of an SDS polyacrylamide gel. Cells which contain the empty vector pSYE263 (lane 1), pSYE263/CYP3A4_na plasmid (lane 2), and pSYE263/CYP3A4_yc (lane 3). The expected size of CYP3A4 protein is 57.3 kDa. The blot was probed by a human CYP3A4 specific monoclonal antibody (Santa Cruz Biotechnology, Cat no: sc-27639).

The results shown in Figure 3.10 do indicate that the *CYP3A4_yc* gene produces more CYP3A4 protein than the *CYP3A4_na* gene. Densitometric quantification (results not shown) suggests that ~4 times more protein is produced by *CYP3A4_yc* gene than by the native gene isolated from a human liver cDNA library. For all Western blots shown in this thesis, the housekeeping protein actin was used as a control. Actin blots showed equal amounts of actin in all yeast strains used for Western blotting (results not shown).

3.5 Cloning of *CYP2C9_yc* gene in the episomal plasmid pSY263 for expression of human CYP2C9 enzyme

3.5.1 Cloning of *CYP2C9_yc* gene in the episomal plasmid pSY263

CYP2C9 is another one of the ten to twelve CYP enzymes which are widely used for Drug Metabolism studies during the process of drug development (Zhang et al., 2013). The human *CYP2C9_yc* gene, with yeast biased codons, was cloned in the yeast expression vector pSYE263 (Figure 3.1) in a way that was similar to the cloning of the *CYP3A4_yc* gene described in Section 3.4.1.

The resultant plasmid was named pSYE263/h_CYP2C9_yc (Figure 3.12).

A 1485 bp *BamH*I-*Xba*I *CYP2C9_yc* gene fragment (Figure 3.11) was isolated from the plasmid pUC57/BamHI-XbaI/h_CYP2C9_yc for further ligation (mediated by DNA ligase) to the 5699 bp *BamH*I-*Xba*I fragment of vector pSYE263 (Figure 3.5), to create the episomal, 2µ-plasmid pSYE263/h_CYP2C9_yc (Figure 3.12) for expression of the human CYP2C9 enzyme.

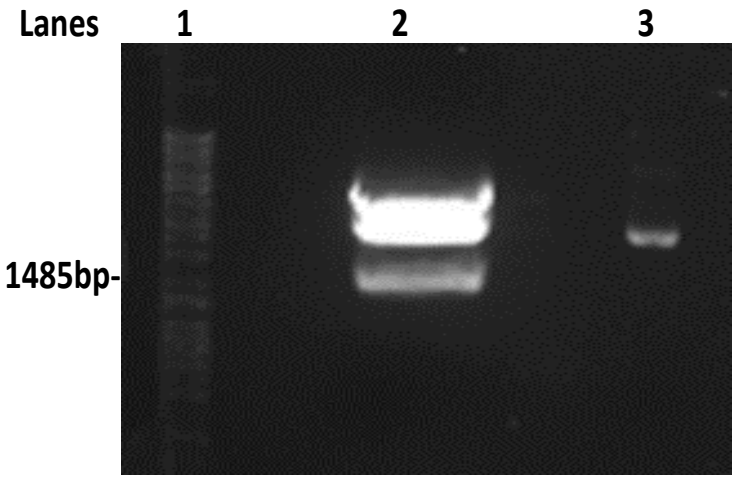


Figure 3.11. The agarose gel shows fractionation of the DNA fragments obtained after digestion of the plasmid pUC57/BamHI-XbaI/h_CYP2C9_yc with *BamH*I and *Xba*l. Lane 1, DNA ladder with defined sizes of DNA fragments; lane 2, pUC57/BamHI-XbaI/h_CYP2C9_yc digested with *BamH*I, *Xba*l; lane 3, uncut plasmid pUC57/BamHI-XbaI/h_CYP2C9_yc. The lower 1485 bp band (lane 2) was isolated from the gel for further ligation with the vector pSYE263, digested with *BamH*I and *Xba*l to obtain the plasmid pSYE263/h_CYP2C9_yc.

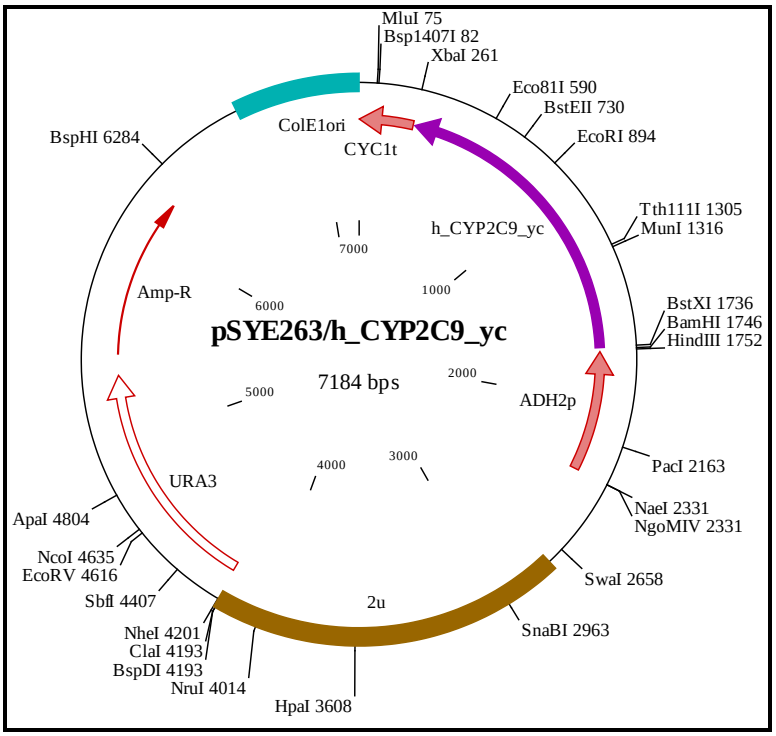


Figure 3.12. Shows the plasmid map of pSYE263/h_CYP2C9_yc, with restriction sites that occur in the plasmid only once. The *BamH*I and *Xba*I sites of pSYE263 were used for cloning the synthetic *h_CYP2C9_yc* gene with yeast biased codons, in the episomal, 2µ-plasmid pSYE263.

After ligation and transformation of ligation mixtures in *E. coli* DH5α, individual colonies were grown for preparation of plasmid DNA using the alkaline lysis method (Chapter 2, Section 2.4.1.8).

The authenticity of the resultant plasmid DNA, pSYE263/h_CYP2C9_yc was confirmed by digesting with the restriction enzymes *BamH*I and *Xba*I. It was verified that the correct size insert (1485 bp) existed within the newly constructed plasmid (Figure 3.13).

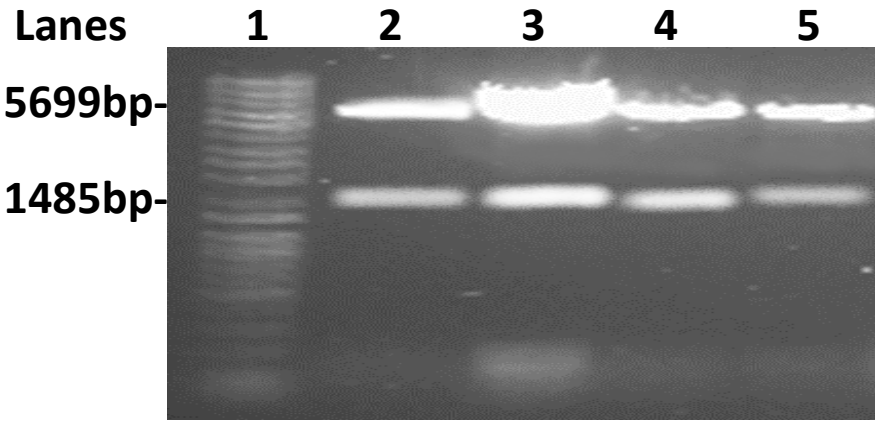


Figure 3.13. An agarose gel that shows the expected DNA fragments when the plasmid pSYE263/h_CYP2C9_yc was digested with the restriction enzymes BamHI and XbaI. Lane 1, DNA ladder; lanes 2 to 5, pSYE263/h_CYP2C9_yc digested with BamHI and XbaI.

3.5.2 Comparison of CYP2C9 enzyme activities expressed by 'synthesised' *CYP2C9_yc* and 'native' *CYP2C9_na* genes, both encoded by the episomal plasmid pSY263, using a fluorescence-based assay

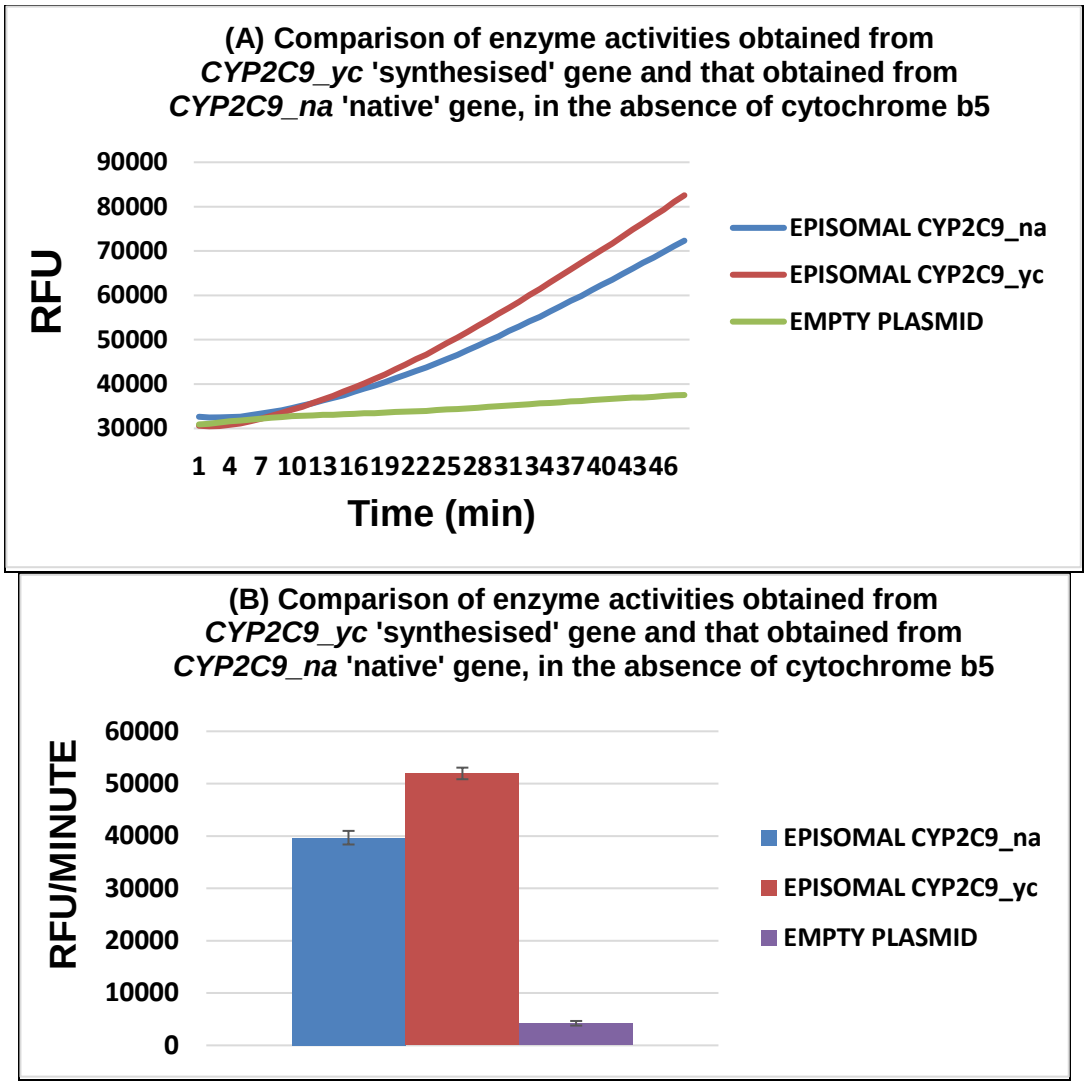


Figure 3.14. (A) The graphs show the rate at which the activity of CYP2C9 enzyme, expressed in yeast from the 'synthesised' *CYP2C9_yc* gene and the 'native' *CYP2C9_na* gene (co-expressed only with the human CPR gene, ΔhRDM but in the absence of cytochrome b5), increases over 50 min. Rate was measured in terms of relative fluorescence units (RFUs), using CEC (3-cyano-7-ethoxycoumarin) as the substrate. CEC is de-alkylated by CYP2C9 to form the fluorescent product 3-cyano-7-hydroxycoumarin (CHC). The empty plasmid pSYE263 (containing no CYP2C9 gene), when expressed in yeast, showed basal levels of activity which equates to no activity (green). (B) Depicts the comparison of fluorescence emitted by the three yeast strains at 45 min of reaction of enzyme with substrate. The data represent mean ± S.D. of three independent experiments.

The results in Figure 3.14 (shown above) would indicate that there is slightly more protein expressed from the 'synthesised' *CYP2C9_yc* gene than from the 'native' *CYP2C9_na* gene. Again, as expected the gene with the yeast biased codons provides mRNA more stable than transcribed by the 'native' gene. This would translate to more protein relative to what would be produced by the 'native' mRNA. The results also show that active CYP2C9 can be expressed from yeast, in the absence of cytochrome b5. Recently, Corning-Gentest (the leading manufacturer of human enzymes in insect cells) have started distributing human CYP2C9 microsomal enzyme which has been produced in the absence of cytochrome b5.

The results in Figure 3.14 was also confirmed by performing Western blot analysis using equal number of cells (1X 10^6) that express the two variants, 'synthesised' and 'native', of the *CYP2C9* gene (Figure 3.15).

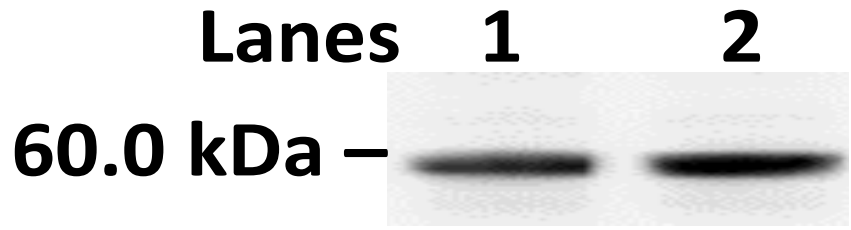


Figure 3.15. Western blot shows CYP2C9 proteins expressed from equal number of cells (1X 10^6). The total cellular protein obtained from these cells was loaded on wells of an SDS polyacrylamide gel. Lane 1, cells expressing human CYP2C9 protein from a 'synthetic' gene; Lane 2, cells expressing human CYP2C9 protein from a 'native' CYP2C9 gene. The expected size of CYP2C9 protein is 55.6 kDa. The blot was probed by a human CYP2C9 specific monoclonal antibody (Santa Cruz Biotechnology, Cat no: sc-374421).

Densitometric measurements (results not shown) of the blot shown in Figure 3.15 indicate that there is ~2 times more protein obtained from *CYP2C9_yc* gene than from the *CYP2C9_na* gene.

3.6 Cloning of *CYP2C19_yc* gene in the episomal plasmid pSY263 for expression of human CYP2C19 enzyme

3.6.1 Cloning of *CYP2C19_yc* gene in the episomal plasmid pSY263

CYP2C19 is another CYP enzyme, which is widely used for Drug Metabolism studies in the process of drug development (Zanger et al., 2013). The human *CYP2C19_yc* gene, with yeast biased codons, was cloned in the yeast expression vector pSYE263 using methodologies similar to cloning of the *CYP3A4_yc* gene in the same vector (this Chapter, Section 3.4.1). The resultant plasmid was named pSYE263/h_CYP2C19_yc.

A 1485 bp *BamH*I-*Xba*I *CYP2C19_yc* gene fragment (Figure 3.16) was isolated from the plasmid pUC57/BamHI-XbaI/h_CYP2C19_yc for DNA ligase mediated ligation to the 5699 bp *BamH*I-*Xba*I fragment of vector pSYE263 (Figure 3.5), to create the episomal, 2µ-plasmid pSYE263/h_CYP2C19_yc (Figure 3.17) for expression of human CYP2C19 enzyme.

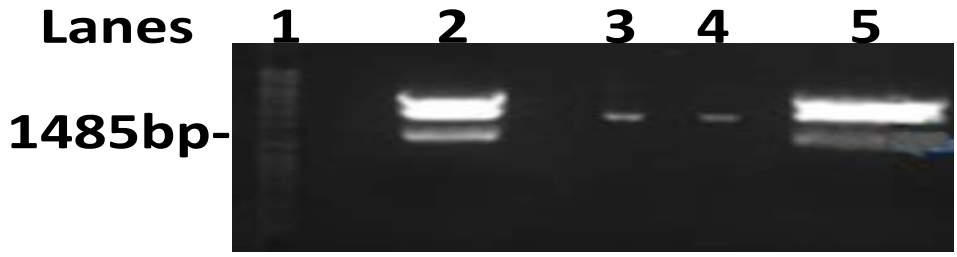


Figure 3.16. The agarose gel shows fractionation of the DNA fragments obtained after digestion of the plasmid pUC57/BamHI-XbaI/h_CYP2C19_yc with BamHI and Xbal. Lane 1, DNA ladder with defined sizes of DNA fragments; lanes 2 & 5, pUC57/BamHI-XbaI/h_CYP2C19_yc digested with BamHI, Xbal; lanes 3 & 4, uncut plasmid pUC57/BamHI-XbaI/h_CYP2C19_yc. The lower 1485 bp band (from lanes 2 & 5) was isolated from the gel for further ligation with the vector pSYE263, digested with BamHI and Xbal to obtain the plasmid pSYE263/h_CYP2C19_yc.

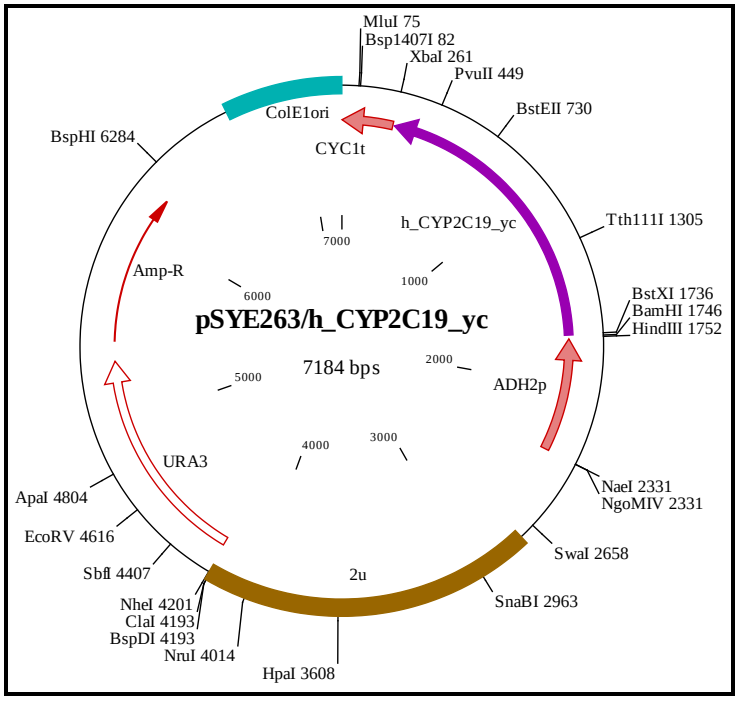


Figure 3.17. Shows the plasmid map of pSYE263/h_CYP2C19_yc, with restriction sites that occur in the plasmid only once. The *BamH*I and *Xba*I sites of pSYE263 were used for cloning the synthetic *h_CYP2C19_yc* gene with yeast biased codons, in the episomal, 2µ-plasmid pSYE263.

After ligation and transformation of the ligation mixture in *E. coli* DH5α competent cells, individual bacterial transformants were grown for preparation of plasmid DNA using the alkaline lysis method (Chapter 2, Section 2.4.1.8).

The authenticity of the resultant plasmid DNA, pSYE263/h_CYP2C19_yc was confirmed by digesting with the restriction enzymes *BamH*I and *Xba*I. Thus, it was verified that the correct size insert (1485 bp) existed within the newly constructed plasmid (Figure 3.18).

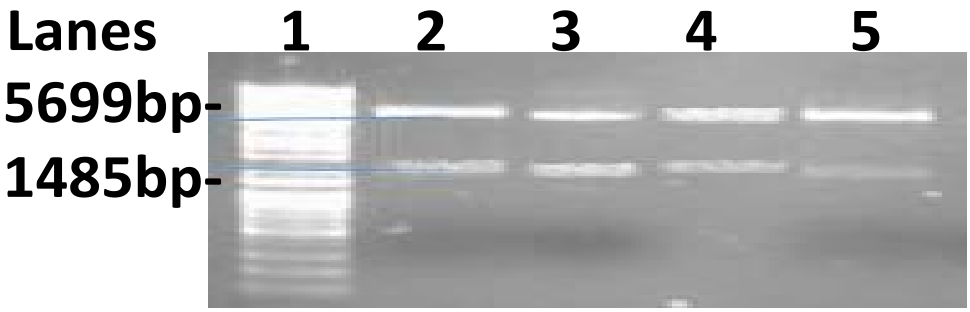


Figure 3.18. An agarose gel that shows the expected DNA fragments when the plasmid pSYE263/h_CYP2C19_yc was digested with the restriction enzymes BamHI and XbaI. Lane 1, DNA ladder; lanes 2 to 5, pSYE263/h_CYP2C19_yc digested with BamHI and XbaI.

3.6.2 Comparison of CYP2C19 enzyme activities expressed by 'synthesised' *CYP2C19_yc* and 'native' *CYP2C19_na* genes, both encoded by the episomal plasmid pSY263, using a fluorescence-based assay

The results shown in Figure 3.19 (which follows) indicate that there is much more protein expressed from the 'synthesised' *CYP2C19_yc* gene than from the 'native' *CYP2C19_na* gene because. Once again, the gene with the yeast biased codons should provide mRNA which is more stable than the mRNA transcribed by the 'native' gene.

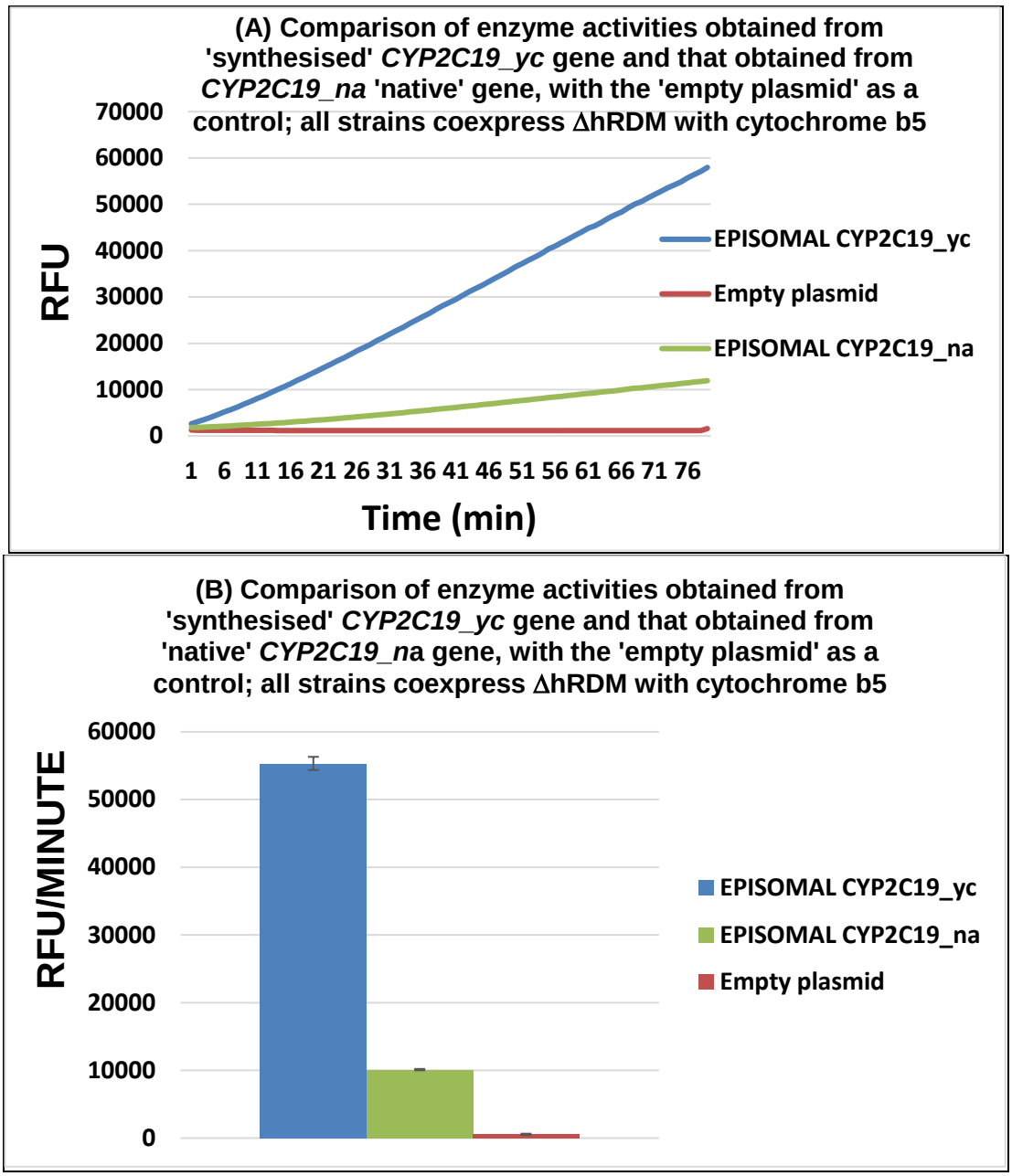


Figure 3.19. (A) The graphs show the rate at which the activity of the CYP2C19 enzyme, expressed in yeast from the 'synthesised' *CYP2C19_yc* gene and the 'native' *CYP2C19_na* gene (co-expressed with a modified human *CPR* and cytochrome *b5* genes), increases over 75 min. Rate was measured in terms of relative fluorescence units (RFUs), using 3-cyano-7-ethoxycoumarin (CEC) as the substrate. CEC is de-ethylated by CYP2C19 to form 3-cyano7-hdroxycoumarin (CHC). The empty plasmid pSYE263 (containing no *CYP2C19* gene), when expressed in yeast, showed no activity (brown). (B) Depicts the comparison of fluorescence emitted by the three yeast strains at 75 min of reaction of enzyme with substrate.The data represent mean ± S.D. of three independent experiments.

The mRNA derived from yeast biased codons *CYP2C19* coding sequence should translate to more protein relative to what would be produced by the 'native' mRNA. This would have to be confirmed by Western blotting, using equal number of cells (1X 10^6), that express the two variants, 'synthesised' and 'native', of the *CYP2C19* gene (Figure 3.20).

Figure 3.20. Western blot that shows the amounts of CYP2C19 protein being expressed by equal number of cells (1X 10^6) from different yeast strains. The total cellular protein obtained from these cells was loaded on wells of an SDS polyacrylamide gel. Cells which contain the empty vector pSYE263 (lane 1), pSYE263/CYP2C19_na plasmid (lane 2), and pSYE263/CYP2C19_yc plasmid (lane 3). The expected size of CYP2C19 protein is 55.9 kDa. The blot was probed by a human CYP2C19 specific monoclonal antibody (Santa Cruz Biotechnology, Cat no:sc25581).

The results shown in Figure 3.20 would indicate that the *CYP2C19_yc* gene indeed produces more CYP2C19 protein than the *CYP2C19_na* gene. Densitometric quantification (results not shown) suggests that ~5 times more protein is produced by *CYP2C19_yc* gene than by the native *CYP2C19_na* gene isolated from a human liver cDNA library.

3.7 Cloning of *CYP2B6_yc* gene in the episomal plasmid pSY263 for expression of human CYP2B6 enzyme

3.7.1 Cloning of *CYP2B6_yc* gene in the episomal plasmid pSY263

CYP2B6 is another CYP enzyme which is widely used for Drug Metabolism studies in the process of drug development (Zanger et al., 2013). The human *CYP2B6_yc* gene, with yeast biased codons, was cloned in the yeast expression vector pSYE263 using methodologies similar to cloning of the *CYP3A4_yc* gene in the same vector (this Chapter, Section 3.4.1). The resultant plasmid was named pSYE263/h_CYP2B6_yc.

A 1491 bp *BamH*I-*Xba*I *CYP2B6_yc* gene fragment (Figure 3.21) was isolated from the plasmid pUC57/BamHI-XbaI/h_CYP2B6_yc for DNA ligase mediated ligation to the 5699 bp *BamH*I-*Xba*I fragment of vector pSYE263 (Figure 3.5), to create the episomal, 2µ-plasmid pSYE263/h_CYP2B6_yc (Figure 3.22) for expression of human CYP2B6 enzyme.

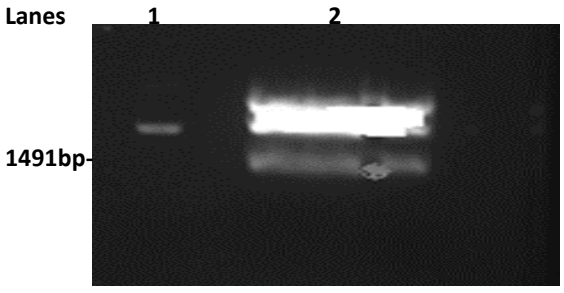


Figure 3.21. An agarose gel that shows the fractionation of DNA fragments obtained after digestion of the plasmid pUC57/BamHI-XbaI/h_CYP2B6_yc with enzymes BamHI and Xbal. Lane 1, uncut plasmid pUC57/BamHI-XbaI/h_CYP2B6_yc; lane 2, pUC57/BamHI-XbaI/h_CYP2B6_yc digested with BamHI, Xbal. The lower 1491 bp band (from lane 2) was isolated from the gel for further ligation with the vector pSYE263, digested with BamHI and Xbal to obtain the plasmid pSYE263/h_CYP2B6_yc.

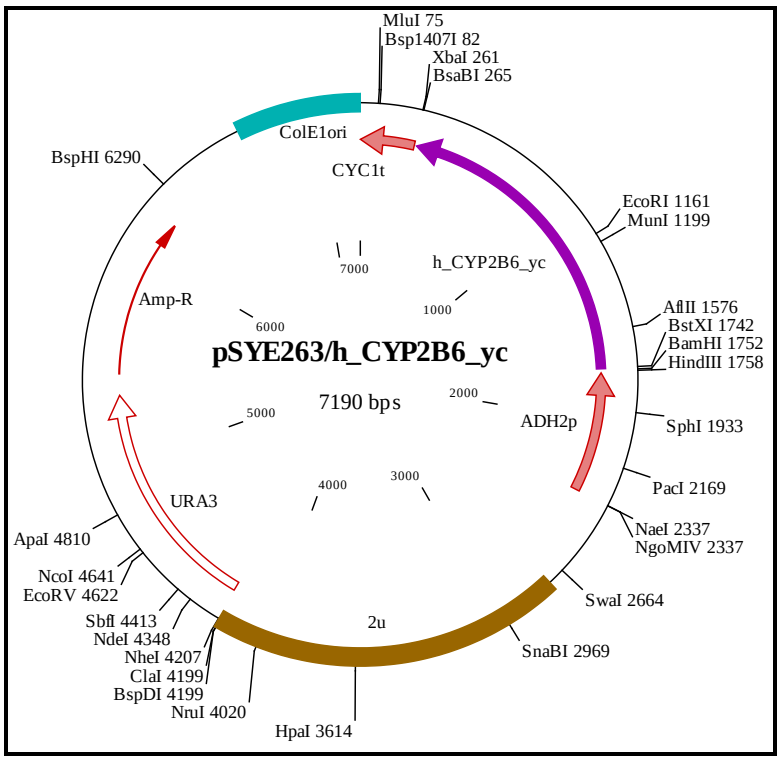


Figure 3.22. The map of plasmid pSYE263/h_CYP2B6_yc, with restriction sites that occur in the plasmid only once. The *BamH*I and *Xba*I sites of pSYE263 were used for cloning the synthetic *h_CYP2B6_yc* gene with yeast biased codons, in the episomal, 2µ-plasmid pSYE263.

After ligation and transformation of the ligation mixture in *E. coli* DH5α competent cells, individual bacterial transformants were grown for preparation of plasmid DNA using the alkaline lysis method (Chapter 2, Section 2.4.1.8).

The authenticity of the resultant plasmid DNA, pSYE263/h_CYP2B6_yc was confirmed by digesting with the restriction enzymes *BamH*I and *Xba*I. It was verified that the newly constructed plasmid contained the correct size insert (1491 bp; Figure 3.23).

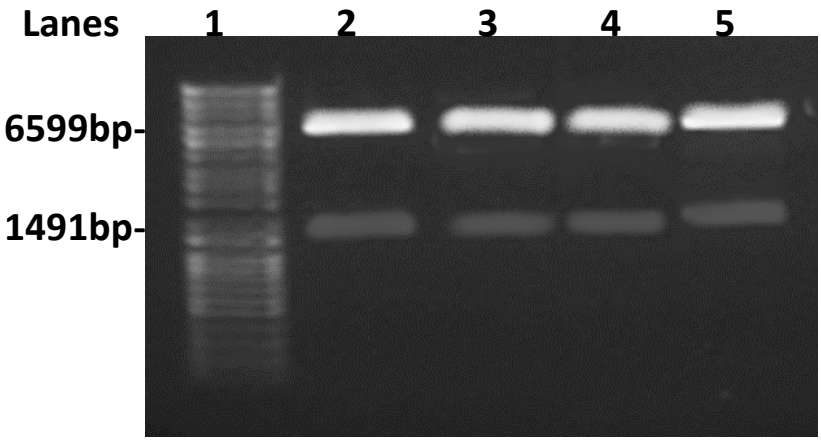
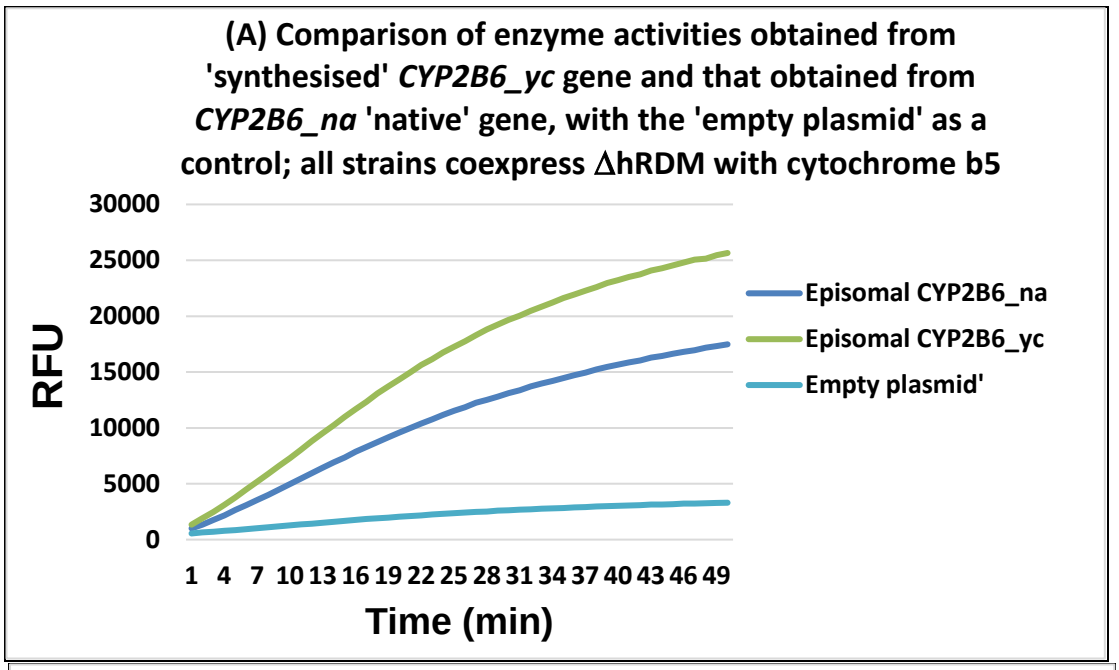


Figure 3.23. An agarose gel that shows the expected DNA fragments when the plasmid pSYE263/h_CYP2B6_yc was digested with the restriction enzymes BamHI and XbaI. Lane 1, DNA ladder; lanes 2 to 5, pSYE263/h_CYP2B6_yc digested with BamHI and XbaI.

3.7.2 Comparison of CYP2B6 enzyme activities expressed by 'synthesised' *CYP2B6_yc* and 'native' *CYP2B6_na* genes, both encoded by the episomal plasmid pSY263, using a fluorescence-based assay

The results shown in Figure 3.24 (that follows) indicate that there is more protein expressed from the 'synthesised' *CYP2B6_yc* gene than from the 'native' *CYP2B6_na* gene because. The gene with the yeast biased codons provides mRNA which is likely to be more stable than the mRNA transcribed by the 'native' gene. The mRNA derived from yeast biased codons *CYP2B6* coding sequence would translate to more protein relative to what would be produced by the 'native' mRNA. This would have to be confirmed by Western blotting, using equal number of cells (1X 10^6), that express the two variants, 'synthesised' and 'native', of the *CYP2B6* gene (Figure 3.25).



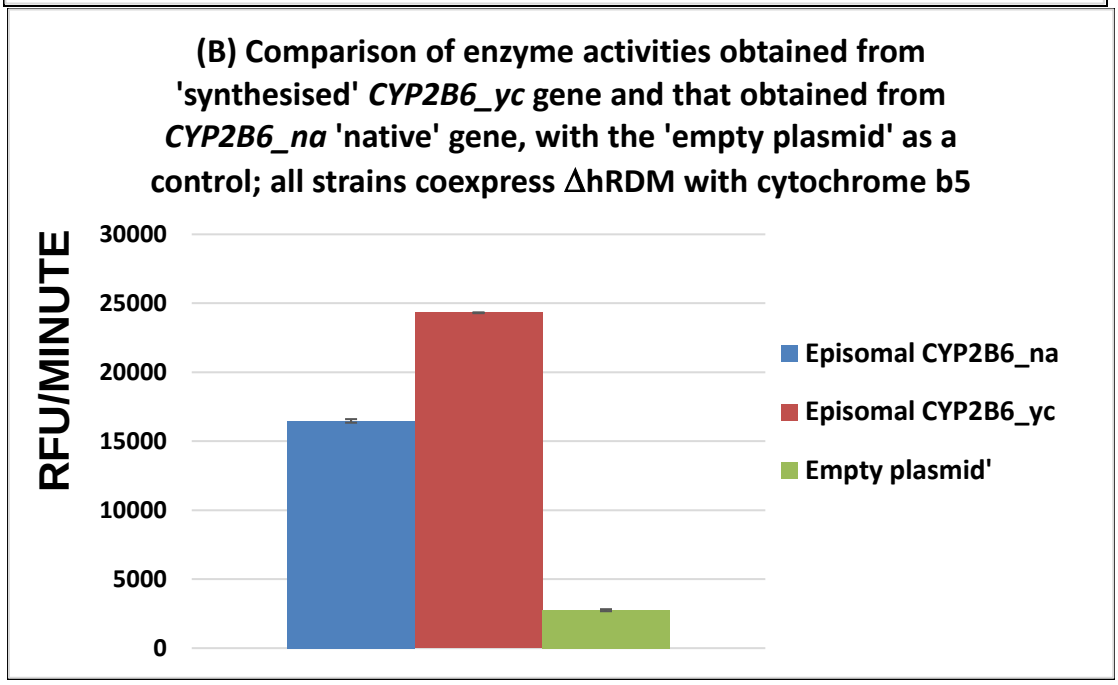


Figure 3.24. (A) The graphs show the rate at which the activity of the CYP2B6 enzyme, expressed in yeast from the 'synthesised' *CYP2B6_yc* gene and the 'native' *CYP2B6_na* gene (co-expressed with a modified human *CPR* and cytochrome *b5* genes), increases over 50 min. Rate was measured in terms of relative fluorescence units (RFUs), using dibenzylfluorescein (DBF) as substrate. DBF is de-alkylated by CYP2B6 enzyme to form the fluorescent product flourscein. The empty plasmid pSYE263 (containing no *CYP2B6* gene), when expressed in yeast, showed no activity (brown). (B) Depicts the comparison of fluorescence emitted by the three yeast strains at 50 min of reaction of enzyme with substrate. The data represent mean ± S.D. of three independent experiments.

The results in Figure 3.24 would indicate that there is more protein expressed from the 'synthesized' *CYP2B6_yc* gene than from the 'native' *CYP2B6_na* gene because, as would be expected, the gene with the yeast biased codons would provide a more stable mRNA (i.e. more mRNA relative to what would be transcribed from the 'native' gene) which would translate to more protein (relative to what would be produced by the 'native' mRNA). This would have to be confirmed by performing Western blot analysis on equal number of cells that express the two variants ('synthesized' and 'native') of the *CYP2B6* gene.

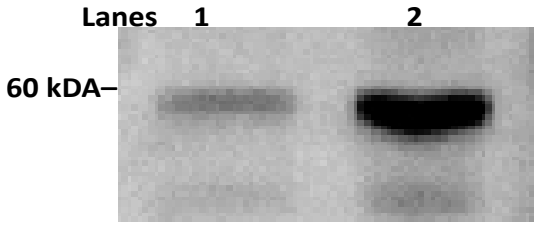


Figure 3.25. Western blot that shows the amounts of CYP2B6 protein being expressed by equal number of cells (1X 10^6) from different yeast strains. The total cellular protein obtained from these cells was loaded on wells of an SDS polyacrylamide gel. Cells which contain pSYE263/CYP2B6_na plasmid (lane 1), and pSYE263/CYP2B6_yc (lane 2). The expected size of CYP2B6 protein is 56.3 kDa. The blot was probed by a human CYP2B6 specific monoclonal antibody (Santa Cruz Biotechnology, Cat no: sc18852).

The results shown in Figure 3.25 indicate that indeed the *CYP2B6_yc* gene produces more CYP2B6 protein than the *CYP2B6_na* gene. Densitometric quantification (results not shown) suggests that at least 6 times more protein is produced by *CYP2B6_yc* gene than by the native *CYP2B6_na* gene isolated from a human liver cDNA library. We are not sure why this is not reflected in the enzyme activity results shown in Figure 3.24;

however, the microsomal CYP2B6 enzyme isolated from strain expressing *CYP2B6_yc* does show more activity than enzyme isolated from the strain expressing *CYP2B6_na* (data not shown).

3.8 Cloning of *CYP2C18_yc* gene in the episomal plasmid pSY263 for expression of human CYP2C18 enzyme

3.8.1 Cloning of *CYP2C18_yc* gene in the episomal plasmid pSY263

CYP2C18 is another one of the CYP enzymes which is used for Drug Metabolism studies during the process of drug development (Meyer et al., 2010). The human *CYP2C18_yc* gene, with yeast biased codons, was cloned in the yeast expression vector pSYE263 using methodologies similar to cloning of the *CYP3A4_yc* gene in the same vector (this Chapter, Section 3.4.1). The resultant plasmid was named pSYE263/h_CYP2C18_yc.

A 1488 bp *BamH*I-*Xba*I *CYP2C18_yc* gene fragment (Figure 3.26) was isolated from the plasmid pUC57/BamHI-XbaI/h_CYP2C18_yc for DNA ligase mediated ligation to the 5699 bp *BamH*I-*Xba*I fragment of vector pSYE263 (Figure 3.5), to create the episomal, 2µ-plasmid pSYE263/h_CYP2C18_yc (Figure 3.27) for expression of human CYP2C18 enzyme.

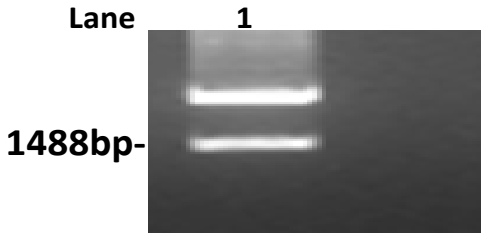


Figure 3.26. Agarose gel showing the fractionation of DNA fragments obtained after digestion of the plasmid pUC57/BamHI-XbaI/h_CYP2C18_yc with

enzymes BamHI and Xbal. Lane 1, pUC57/BamHI-XbaI/h_CYP2B6_yc digested with BamHI, Xbal. The lower 1488 bp band (lane 1) was isolated from the gel for further ligation with the vector pSYE263, digested with BamHI and Xbal to obtain the plasmid pSYE263/h_CYP2C18_yc.

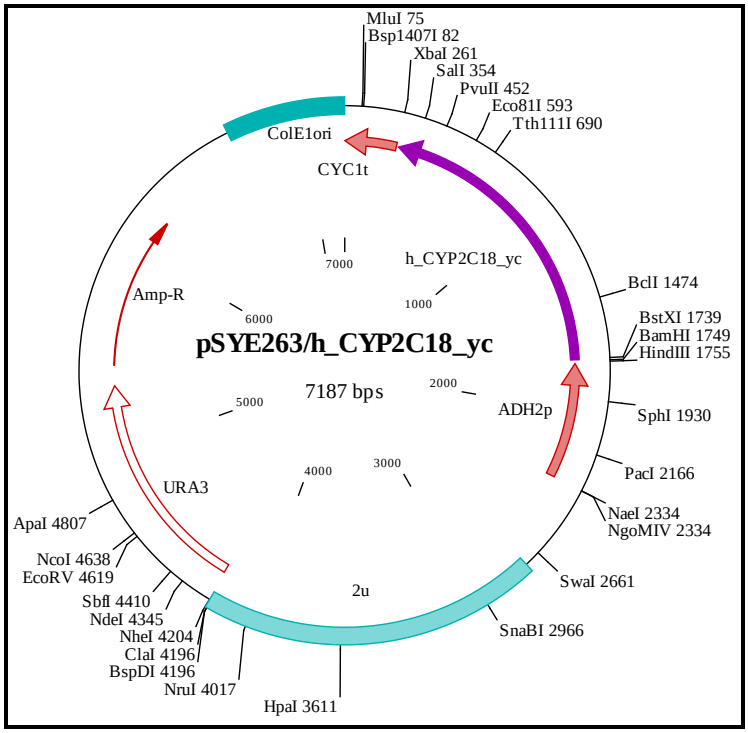


Figure 3.27. The map of plasmid pSYE263/h_CYP2C18_yc, with restriction sites that occur in the plasmid only once. The BamHI and XbaI sites of pSYE263 were used for cloning the synthetic *h_CYP2C18_yc* gene with yeast biased codons, in the episomal, 2µ-plasmid pSYE263.

After ligation and transformation of the ligation mixture in *E. coli* DH5α competent cells, individual bacterial transformants were grown for preparation of plasmid DNA using the alkaline lysis method (Chapter 2, Section 2.4.1.8).

The authenticity of the resultant plasmid DNA, pSYE263/h_CYPC18_yc was confirmed by digesting with the restriction enzymes *BamH*I and *Xba*I. It was verified that the newly constructed plasmid contained the correct size insert (1488 bp; Figure 3.28).

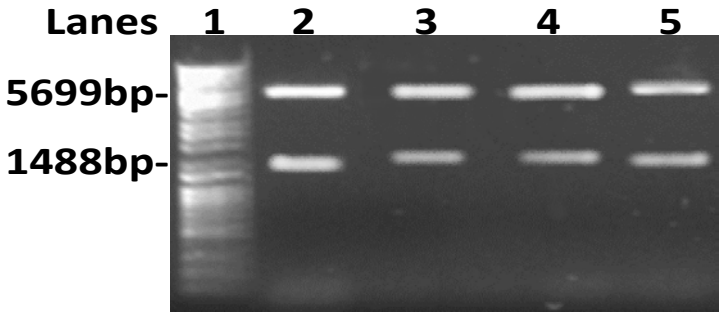


Figure 3.28. An agarose gel showing the expected DNA fragments when plasmid pSYE263/h_CYP2C18_yc was digested with the enzymes BamHI and XbaI. Lane 1, DNA ladder; lanes 2 to 5, pSYE263/h_CYP2C18_yc digested with BamHI and XbaI.

3.8.2 Comparison of CYP2C18 enzyme activities expressed by 'synthesised' *CYP2C18_yc* encoded by the episomal plasmid pSY263, in the presence or absence of cytochrome b5, using a fluorescence-based assay

The results shown in Figure 3.29 (which follows) indicate that roughly similar levels of CYP2C18 enzyme activity are expressed from the 'synthesised' *CYP2C18_yc* gene in the absence or presence of cytochrome b5. Western blotting confirms that the CYP2C18 protein is expressed at similar levels in the absence and presence of cytochrome b5 (Figure 3.30).

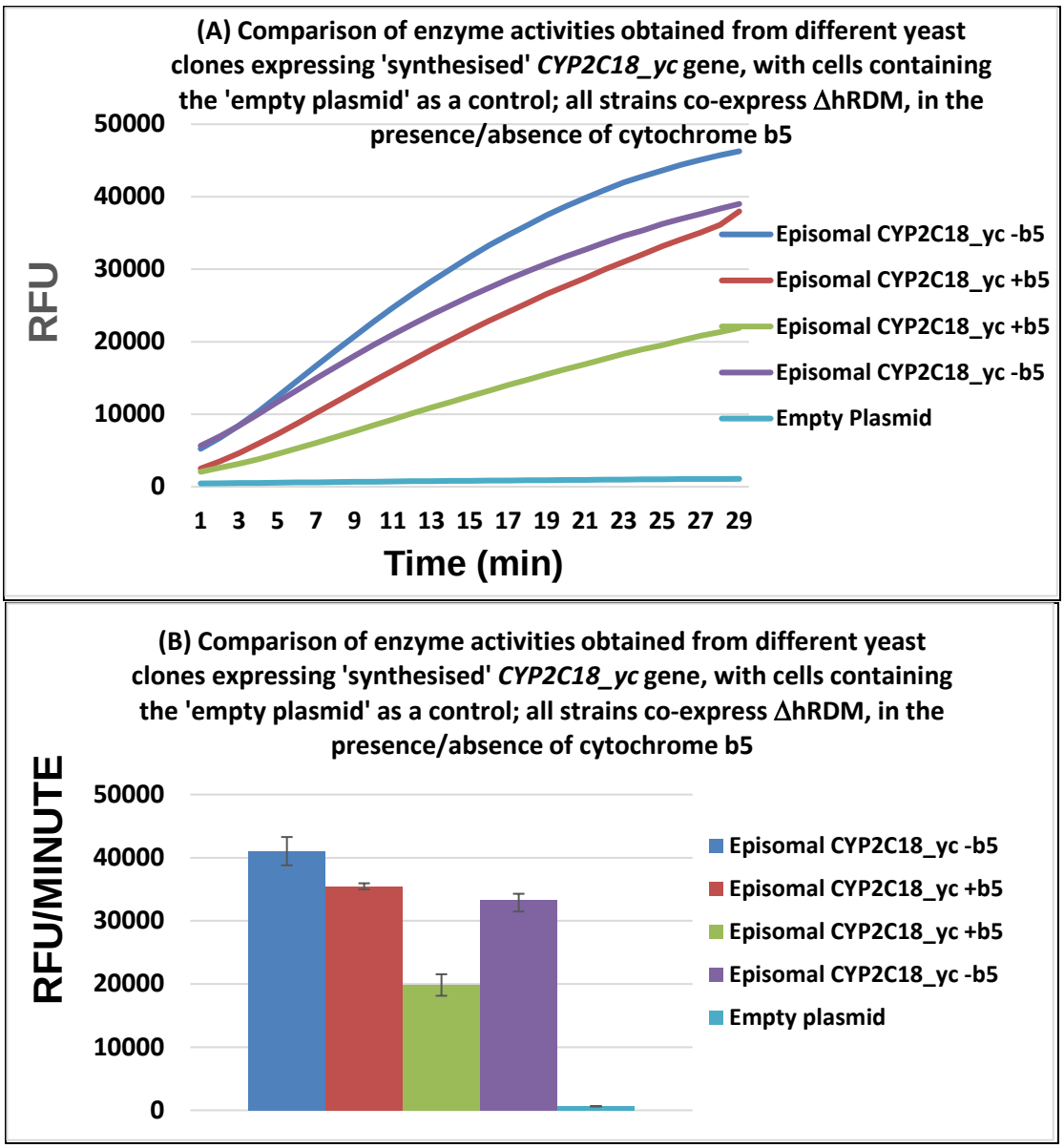


Figure 3.29. (A) The graph shows the rate at which the activity of the CYP2C18 enzyme, expressed in yeast strains from the 'synthesised' *CYP2C18_yc* gene, increases over 30 min. Two of the strains co-express only ΔhRDM but two others co-express not only ΔhRDM but also cytochrome b5. Rate of reactions was measured in terms of relative fluorescence units (RFUs), using dibenzylfluorescein (*DBF*) as the substrate. DBF is dealkylated by CYP2C18 enzyme to form the fluorescent product flourscein. The empty plasmid pSYE263 (containing no *CYP2C18* gene), when expressed in yeast, showed more or less no activity. (B) Depicts the comparison of fluorescence emitted by the yeast strains (in the presence/absence of cytochrome b5) at 30 min of reaction of enzyme with substrate. The data represent mean ± S.D. of three independent experiments.

The results shown in Figure 3.30 suggest that all clones from the CYP2C18-producing yeast strains express similar amounts of CYP2C18 protein, in the presence or absence of cytochrome b5. It appears from Figure 3.29 that cytochrome b5 may not be helpful at all in augmenting CYP2C18 enzyme activity. However, It has been published that cytochrome b5 is essential for activity of CYP2C enzymes, and specifically CYP2C18 (Zhang et al., 2015).

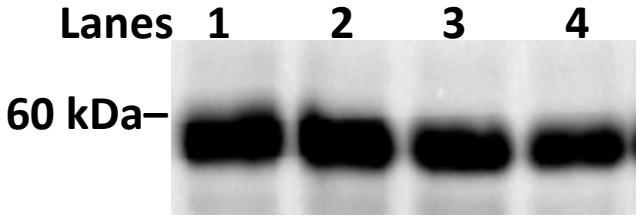


Figure 3.30. Western blot showing that CYP2C18 protein is expressed from cells of all yeast strains containing the *CYP2C18_yc* gene. The total cellular protein obtained from these cells (1X 10^6) was loaded on wells of an SDS polyacrylamide gel. Lanes 1, 2: CYP2C18 protein expressed from cells co-expressing cytochrome b5. Lanes 2, 4: CYP2C18 protein expressed from cells that did not co-express cytochrome b5. The expected size of CYP2C18 protein is 55.9 kDa. The blot was probed by a human CYP2C18 specific monoclonal antibody (Abcam, Cat no: ab175982).

3.9 Cloning of *CYP1A2_yc* gene in the episomal plasmid pSY263 for expression of human CYP1A2 enzyme

3.9.1 Cloning of *CYP1A2_yc* gene in the episomal plasmid pSY263

CYP1A2 is another CYP enzyme which is used for Drug Metabolism studies in the process of drug development (Zanger et al., 2013). The human *CYP1A2_yc* gene, with yeast biased codons, was cloned in the yeast expression vector pSYE263 using methodologies similar to cloning of the *CYP3A4_yc* gene in the same vector (this Chapter, Section 3.4.1). The resultant plasmid was named pSYE263/h_CYP1A2_yc.

A 1563 bp *BamH*I-*Xho*I *CYP1A2_yc* gene fragment (Figure 3.31) was isolated from the plasmid pUC57/BamHI-XhoI/h_CYP1A2_yc for DNA ligase mediated ligation to the 5711 bp *BamH*I-*Xho*I fragment of vector pSYE263, to create the episomal, 2µ-plasmid pSYE263/h_CYP1A2_yc (Figure 3.32) for expression of human CYP1A2 enzyme.

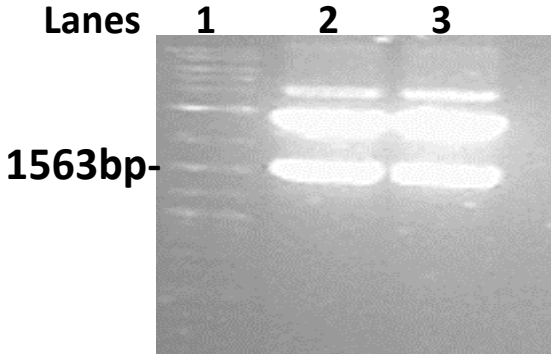


Figure 3.31. An agarose gel that shows the fractionation of DNA fragments obtained after digestion of the plasmid pUC57/BamHI-XhoI/h_CYP1A2_yc with enzymes BamHI and Xhol. Lane 1, DNA ladder of DNA fragments with known molecular weight; lanes 2 and 3, pUC57/BamHI- Xhol/h_CYP1A2_yc digested with BamHI, Xhol. The lower 1488 bp band (from lane 2) was isolated from the gel for further ligation with the vector pSYE263, digested with BamHI and Xhol to obtain the plasmid pSYE263/h_CYP1A2_yc.

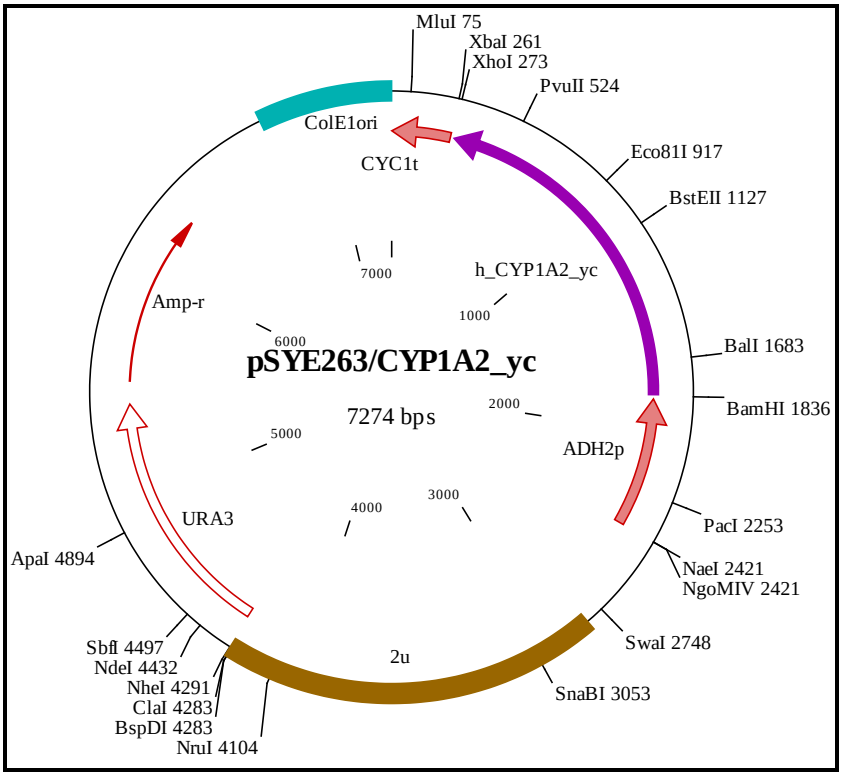


Figure 3.32. The map of plasmid pSYE263/h_CYP1A2_yc, with restriction sites that occur in the plasmid only once. The *BamH*I and *Xho*I sites of pSYE263 were used for cloning the synthetic *h_CYP1A2_yc* gene with yeast biased codons, in the episomal, 2µ-plasmid pSYE263.

After ligation and transformation of the ligation mixture in *E. coli* DH5α competent cells, individual bacterial transformants were grown for preparation of plasmid DNA using the alkaline lysis method (Chapter 2, Section 2.4.1.8)

The authenticity of the resultant plasmid DNA, pSYE263/h_CYP1A2_yc was confirmed by digesting with the restriction enzymes (a) *BamH*I, *Xho*I and (b) *BamH*I, *Xba*I. It was verified that the newly constructed plasmid contained the correct size insert (Figure 3.33).

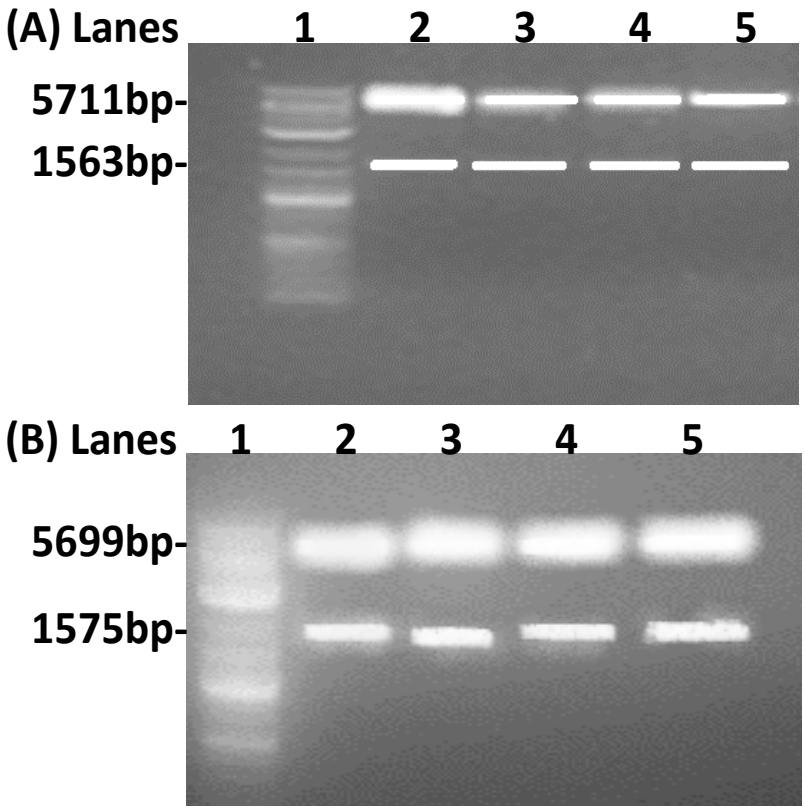


Figure 3.33. Agarose gels that show the expected DNA fragments when the plasmid pSYE263/h_CYP1A2_yc was digested with restriction enzymes (A) *BamH*I, *Xho*I and (B) *BamH*I, *Xba*I. Lane 1, DNA ladder; lanes 2 to 5, pSYE263/h_CYP1A2_yc digested with either *BamH*I-*Xho*I (A) or *BamH*I-*Xba*I (B).

3.9.2 Comparison of CYP1A2 enzyme activities expressed by 'synthesised' *CYP1A2_yc* and 'native' *CYP1A2_na* genes, both encoded by the episomal plasmid pSY263, using a fluorescence-based assay

The results shown in Figure 3.34 (which follows below) indicate that, in the absence of cytochrome b5, there is more CYP1A2 protein expressed from the 'synthesised' *CYP1A2_yc* gene than from the 'native' *CYP1A2_na* gene. The gene with the yeast biased codons should provide mRNA is probably more stable than the mRNA transcribed by the 'native' gene and, thereby, should produce more protein.

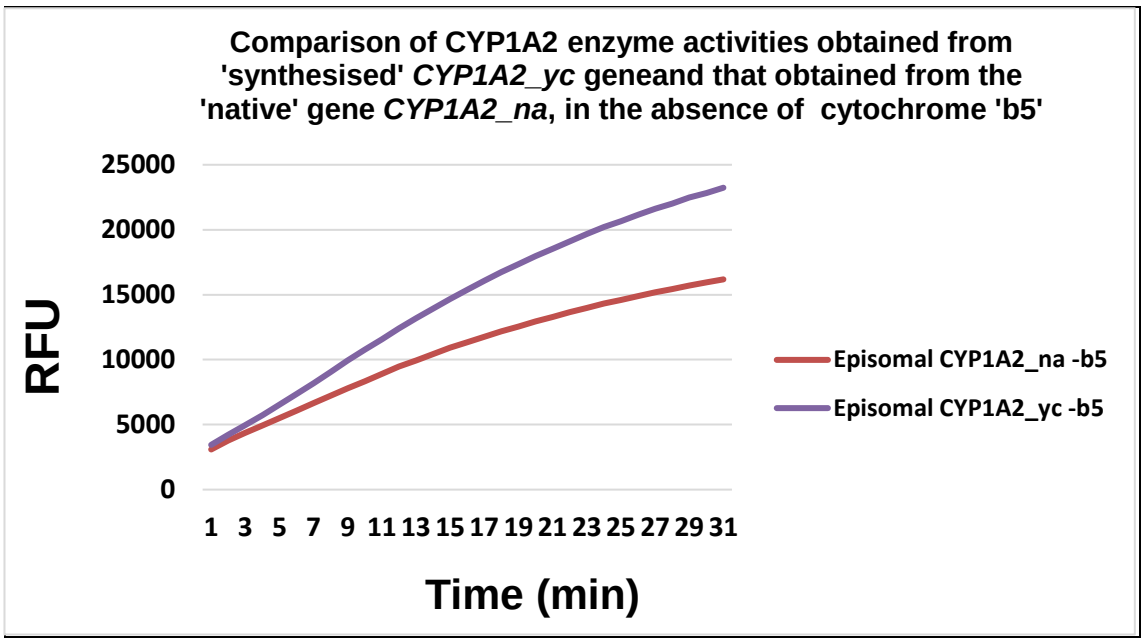
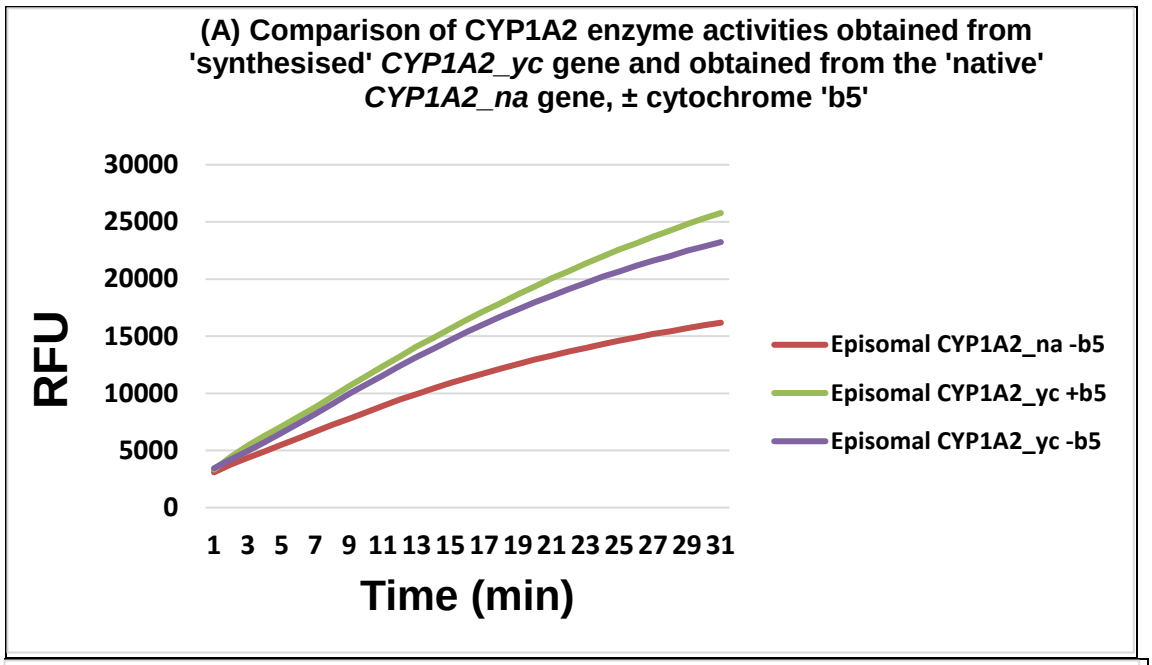


Figure 3.34. The graphs show the rate at which the activity of the CYP1A2 enzyme, expressed in yeast from the 'synthesised' *CYP1A2_yc* gene and the 'native' *CYP1A2_na* gene (co-expressed with only ΔhRDM but in the absence of cytochrome b5), increases over 30 min. Rate was measured in terms of relative fluorescence units (RFUs), using 3-cyano-7-ethoxycoumarin (CEC) as the substrate. CEC is de-ethylated by CYP1A2 to form 3-cyano7-hdroxycoumarin (CHC). The data represent mean ± S.D. of three independent experiments.

The graphs (Figure 3.34) show the rate at which the activity of the CYP1A2 enzyme, expressed from the 'synthesised' *CYP1A2_yc* gene, increases over 30 min (purple) is faster than the rate of the enzyme expressed from the native gene *CYP1A2_na* (red). The results in Figure 3.34 also indicate that there is more protein expressed from the 'synthesized' *CYP1A2_yc* gene than that from the 'native' *CYP1A2_na* gene when expressed in the absence of cytochrome b5.



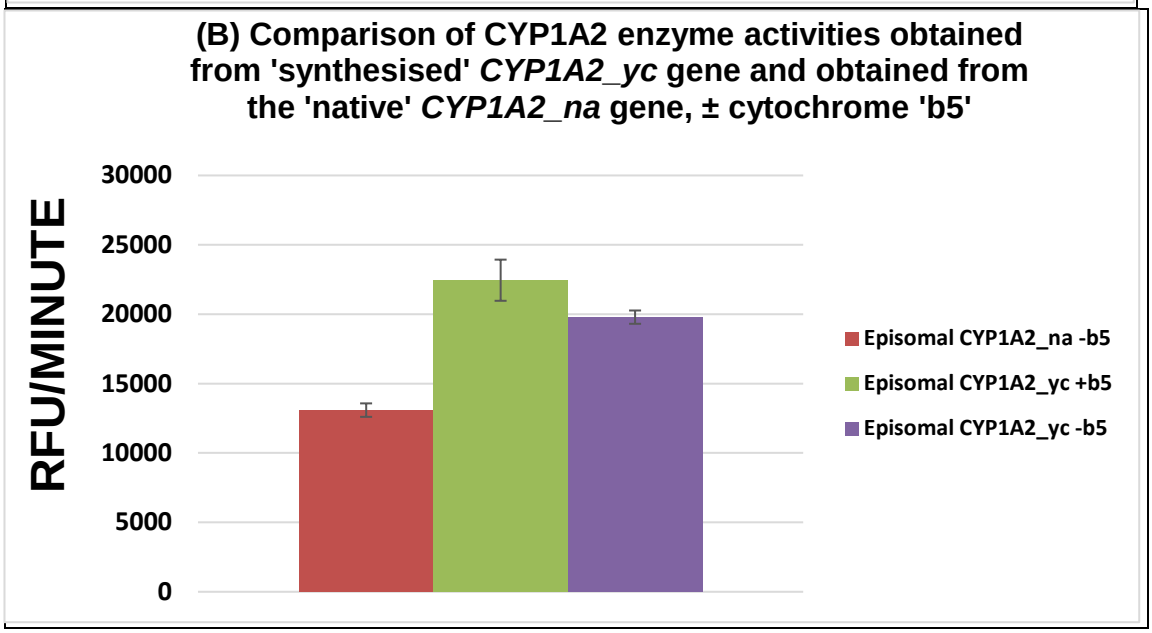


Figure 3.35. (A) The graphs show the increase in rate of activity, over 30 min, of the CYP1A2 enzyme expressed in yeast from the 'synthesised' *CYP1A2_yc* gene which is co-expressed with ΔhRDM in the presence or absence of cytochrome b5. The rates were compared with rate of the enzyme produced from the 'native' *CYP1A2_na* gene co-expressed with only ΔhRDM but not cytochrome b5. Rate was measured in terms of relative fluorescence units (RFUs), using 3-cyano-7-ethoxycoumarin (CEC) as the substrate. CEC is de-ethylated by CYP1A2 to form 3-cyano7-hdroxycoumarin (CHC). (B) Depicts the comparison of fluorescence emitted by the three yeast strains at 30 min of reaction of enzyme with substrate. The data represent mean ± S.D. of three independent experiments.

The results in Figure 3.35 indicate that in the presence of cytochrome b5, slightly more protein is expressed from *CYP1A2_yc* than in its absence. This may suggest cytochrome b5 may provide a protective influence on CYP1A2 protein. This was confirmed by performing Western blot analysis (Figure 3.36) on equal number of cells which express the variants ('synthesized' and 'native') of the *CYP1A2* gene.

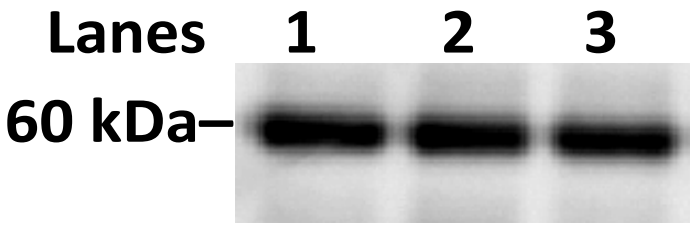


Figure 3.36. Western blot that shows the amounts of CYP1A2 protein which is expressed by equal number of cells (1X 10^6) from different yeast strains. The amount of total cellular protein obtained from these cells was loaded on wells of an SDS polyacrylamide gel. Cells which contain pSYE263/CYP1A2_yc + cytochrome b5 (lane 1), only pSYE263/CYP1A2_yc – cytochrome b5 (lane 2), and only pSYE263/CYP1A2_na – cytochrome b5 (lane 3). The expected size of CYP1A2 protein is 58.4 kDa. The blot was probed by a human CYP1A2 specific monoclonal antibody (Santa Cruz Biotechnology, sc-30085).

The results shown in Figure 3.36 indicate that the *CYP1A2_yc* gene, when co-expressed with cytochrome b5, produces slightly more CYP1A2 protein than when it is expressed in its absence (compare lanes 1 & 2, Figure 3.36). Densitometric quantification (results not shown) shows that there is 50% more CYP1A2 protein, in the presence of cytochrome b5 (lane 1, Figure 3.36), than in its absence (lane 2, Figure 3.36).

3.10 Cloning of four *CYP2D6_yc* variant genes [three bearing single nucleotide polymorphisms (SNPs)] and one the Val^{374} variant in the episomal plasmid pSY263 for expression of human CYP2D6 variant enzymes

3.10.1 Cloning of *CYP2D6_yc* gene in the episomal plasmid pSY263

Like CYP3A4, CYP2D6 is one of the major CYP enzymes which is widely used for Drug Metabolism studies in the process of drug development (Zanger et al., 2013). The human *CYP2D6_yc* gene variants, all synthesized with yeast biased codons, were cloned in the yeast expression vector pSYE263 using methodologies similar to cloning of the *CYP3A4_yc* gene in the same vector (this Chapter, Section 3.4.1). The resultant plasmids had the following names:

(a) pSYE263/h_CYP2D6(1)_yc (bearing Met374Val mutant),

(b) pSYE263/h_CYP2D6*2_yc (bearing the Arg296Cys; Ser486Thr mutant),

(c) pSYE263/h_CYP2D6*10_yc (bearing the Pro34Ser; Ser486Thr mutant),

(d) pSYE263/h_CYP2D6*39_yc (bearing the Ser486Thr mutant).

1506 bp *BamH*I-*Xho*I *CYP2D6_yc* gene fragments [a typical example is provided in Figure 3.37 using the *h_CYP2D6(1)_yc* gene] were isolated from pUC57 based plasmids. A 1506 bp *BamH*I-*Xho*I *CYP2D6(1)_yc* (Figure 3.37) fragment from one such plasmid pUC57/BamHI-XhoI/h_CYP2D6(1)_yc was ligated to the 5711 bp *BamH*I-*Xho*I pSYE263 vector fragment, to create the episomal, 2μ-plasmid

pSYE263/h_CYP2D6(1)_yc (Figure 3.38) for expression of human CYP2D6(1) [Val^{374}] enzyme. Similar approaches were taken to create expression plasmids for the other *CYP2D6* variant genes.

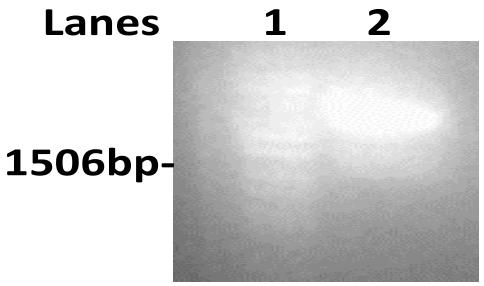


Figure 3.37. An agarose gel that shows the 1506 bp gene insert fragment that was isolated from the plasmid pUC57/BamHI-Xhol/h_CYP2D6(1)_yc after digestion with enzymes *BamH*I-*Xho*l (lane 2). The fragment was isolated for ligation to the pSYE263, vector which had already been digested with *BamH*I-*Xho*l, to obtain the plasmid pSYE263/h_CYP2D6(1)_yc. DNA ladder showing DNA fragments with defined base pairs.

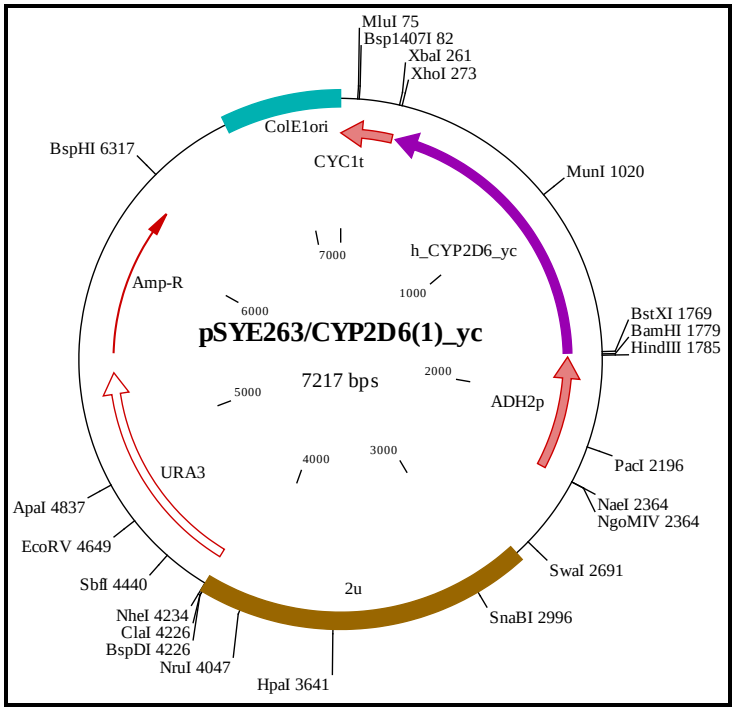


Figure 3.38. The map of plasmid pSYE263/h_CYP2D6(1)_yc, with restriction sites that occur in the plasmid only once. The *BamH*I and *Xho*I sites of the episomal, 2µ-plasmid pSYE263 were used for cloning the synthetic *h_CYP2D6(1)_yc* gene with yeast biased codons.

The authenticity of the resultant plasmid, pSYE263/ BamHI-Xhol/h_CYP2D6(1)_yc was confirmed by two sets of digestions (A) with *BamH*I, *Xhol* and (B) with *BamH*I, *Xba*I restriction enzymes (Figure 3.39).

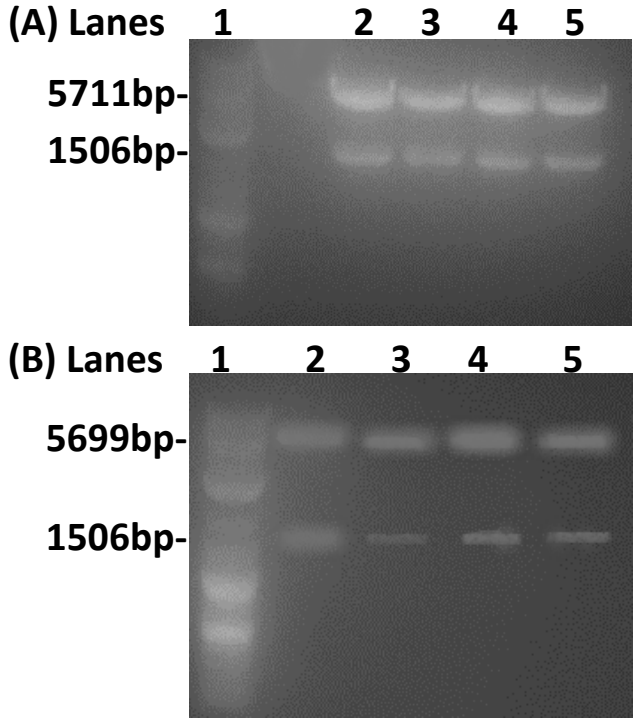


Figure3.39. Agarose gels that show the expected DNA fragments when the plasmid pSYE263/h_CYP2D6(1)_yc was digested with restriction enzymes (A) *BamH*I, *Xho*I and (B) *BamH*I, *Xba*I. Lane 1, DNA ladder; lanes 2 to 5, pSYE263/h_CYP2B6_yc digested with either *BamH*I-*Xho*I (A) or *BamH*I-*Xba*I (B).

Similar sets of experiments, as above, were performed for the other three *CYP2D6* mutants.

3.10.2 Comparison of CYP2D6 enzyme activities expressed by 'synthesised' *CYP2D6_yc* mutant genes, all genes encoded by the episomal plasmid pSY263, using fluorescence-based assays

The results shown in Figures 3.40 to 3.43 (which follow below) indicate that, in the absence of cytochrome b5, there is more CYPD6 protein expressed from the 'synthetic' *CYP2D6_yc* genes (coding for the three different SNPs and the Val^{374} mutant) than from the 'native' *CYP2D6_na* gene. All genes with the yeast biased codons should provide more mRNA because they are more stable than the mRNA transcribed by the 'native' gene and thereby should produce more protein, provided there is no translational block because of the structure of the mRNA.

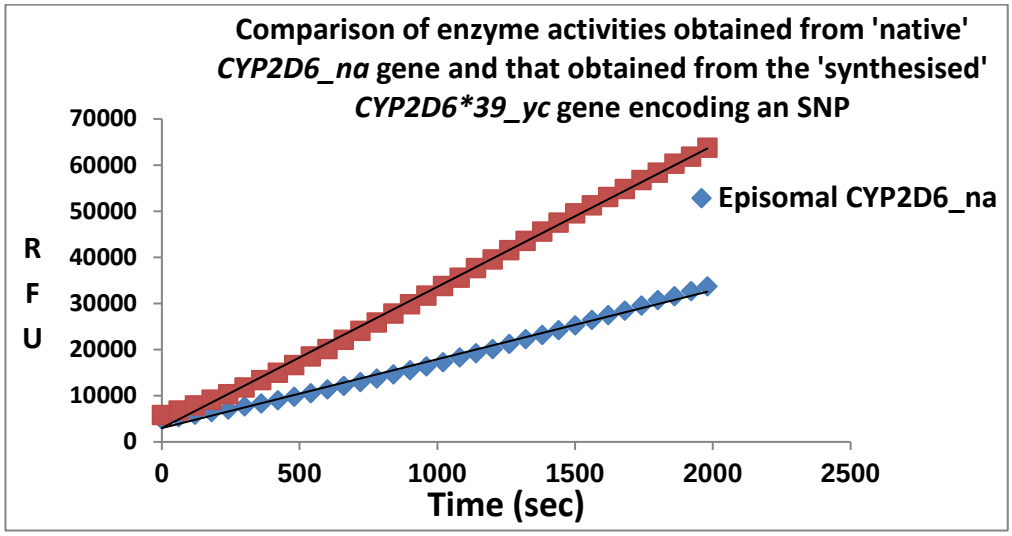


Figure 3.40. The graph compares the rate of reactions of (a) CYP2D6*39 enzyme expressed from a synthetic gene consisting of yeast biased codons, and (b) CYP2D6_na enzyme expressed from the native *CYP2D6* cDNA isolated from the human liver cDNA library. 7-ethoxymethoxy-3-cyanocoumarin (EOMCC) was used as substrate. EOMCC is converted to the fluorescent product 7-hydroxy-3-cyanocoumarin (HCC) upon reaction with CYP2D6. Rate was measured, over 30 min, in terms of relative fluorescence units (RFUs). The data represent mean ± S.D. of three independent experiments.

Both the 'synthesised' *CYP2D6*39_yc* and the native *CYP2D6_na* genes were co-expressed with the modified human *CPR* gene, *ΔhRDM*, but in the absence of cytochrome b5. The results in Figure 3.40 above show that the 'native' *CYP2D6_na* gene when expressed in yeast, possesses relatively lower activity (blue) than the gene encoding SNP *39 (Ton et al., 2004).

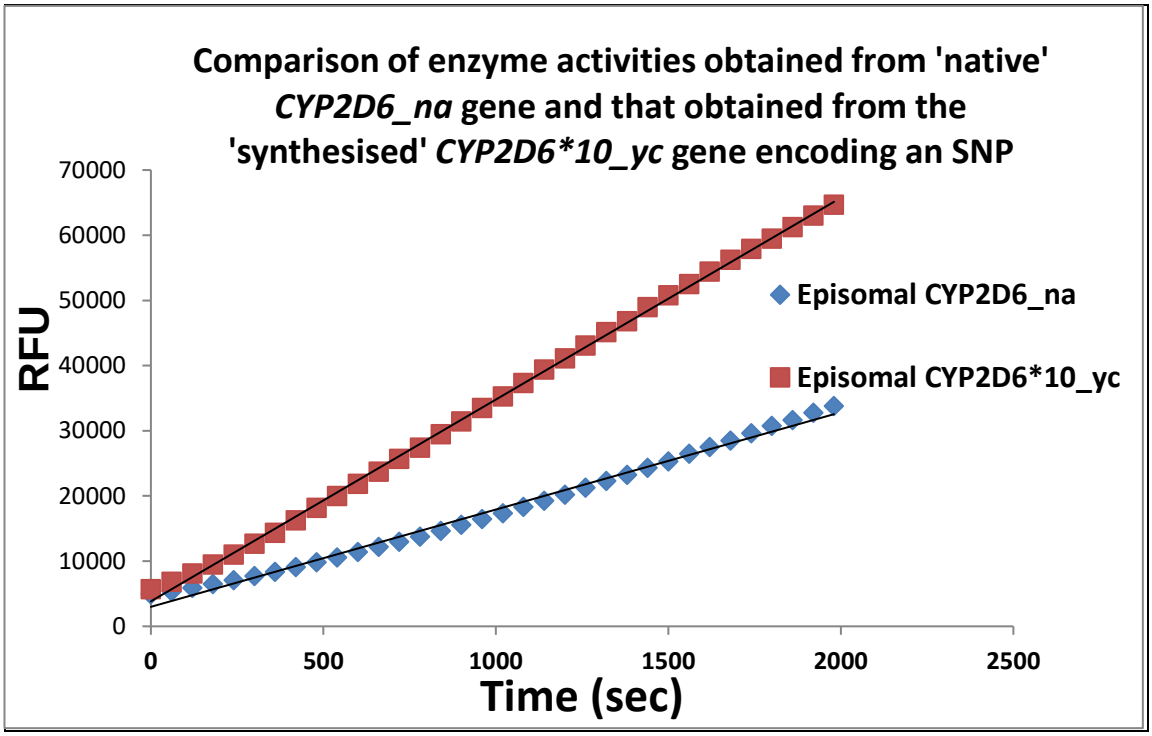


Figure 3.41. The graph compares the rate of reactions of (a) CYP2D6*10 enzyme expressed from a synthetic gene consisting of yeast biased codons, and (b) CYP2D6_na enzyme expressed from the native *CYP2D6* cDNA isolated from the human liver cDNA library. 7-ethoxymethoxy-3-cyanocoumarin (EOMCC) was used as substrate. EOMCC is converted to the fluorescent product 7-hydroxy-3-cyanocoumarin (HCC) upon reaction with CYP2D6. Rate was measured, over 30 min, in terms of relative fluorescence units (RFUs). The data represent mean ± S.D. of three independent experiments.

Both the 'synthesised' *CYP2D6*10_yc* and the native *CYP2D6_na* genes were co-expressed with the modified human *CPR* gene, *ΔhRDM*, without any cytochrome b5. The

results in Figure 3.41 indicate that the 'native' *CYP2D6_na* gene when expressed in yeast, is produced much less (blue) than the gene encoding SNP *10 (Ton et al., 2004).

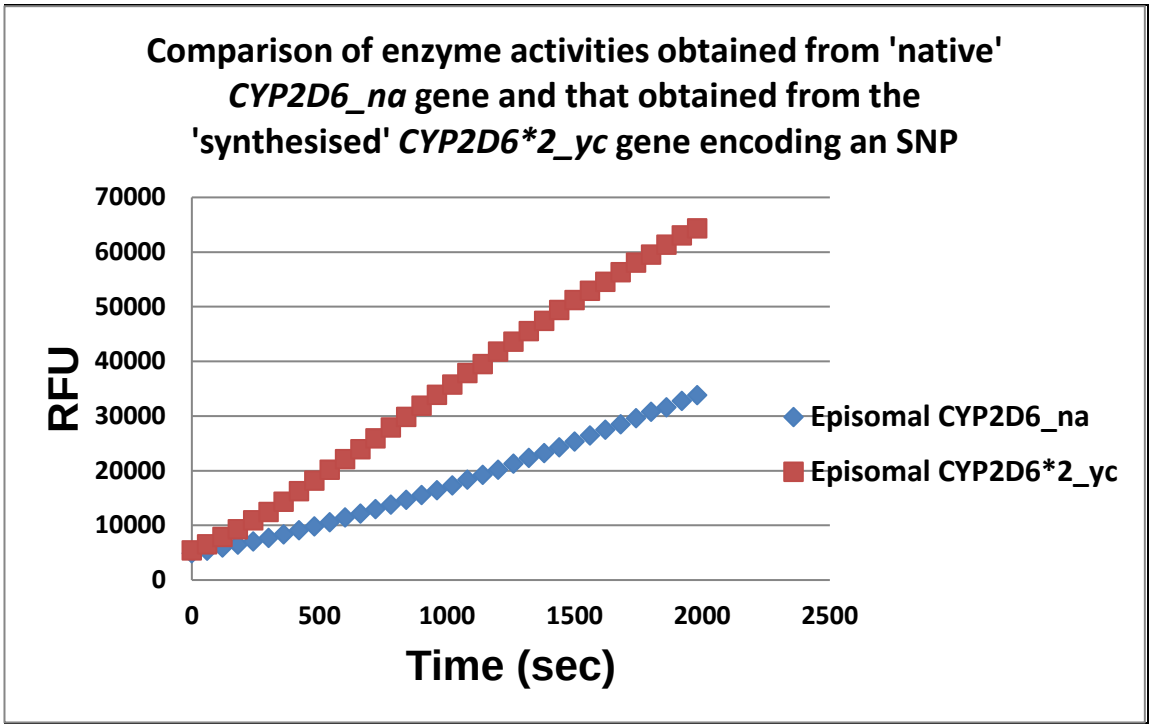


Figure 3.42. The graph compares the rate of reactions of (a) CYP2D6*2 enzyme expressed from a synthetic gene consisting of yeast biased codons, and (b) CYP2D6_na enzyme expressed from the native *CYP2D6* cDNA isolated from the human liver cDNA library. 7-ethoxymethoxy-3-cyanocoumarin (EOMCC) was used as substrate. EOMCC is converted to the fluorescent product 7-hydroxy-3-cyanocoumarin (HCC) upon reaction with CYP2D6. Rate was measured, over 30 min, in terms of relative fluorescence units (RFUs). The data represent mean ± S.D. of three independent experiments.

Both the 'synthesised' *CYP2D6*2_yc* and the native *CYP2D6_na* genes were co-expressed only with the modified human *CPR* gene, *ΔhRDM*, without any cytochrome b5. The results in Figure 3.42 reveal that the 'native' *CYP2D6_na* gene when expressed in yeast, produces less protein (blue) than the gene encoding SNP *2 (Ton et al., 2004).

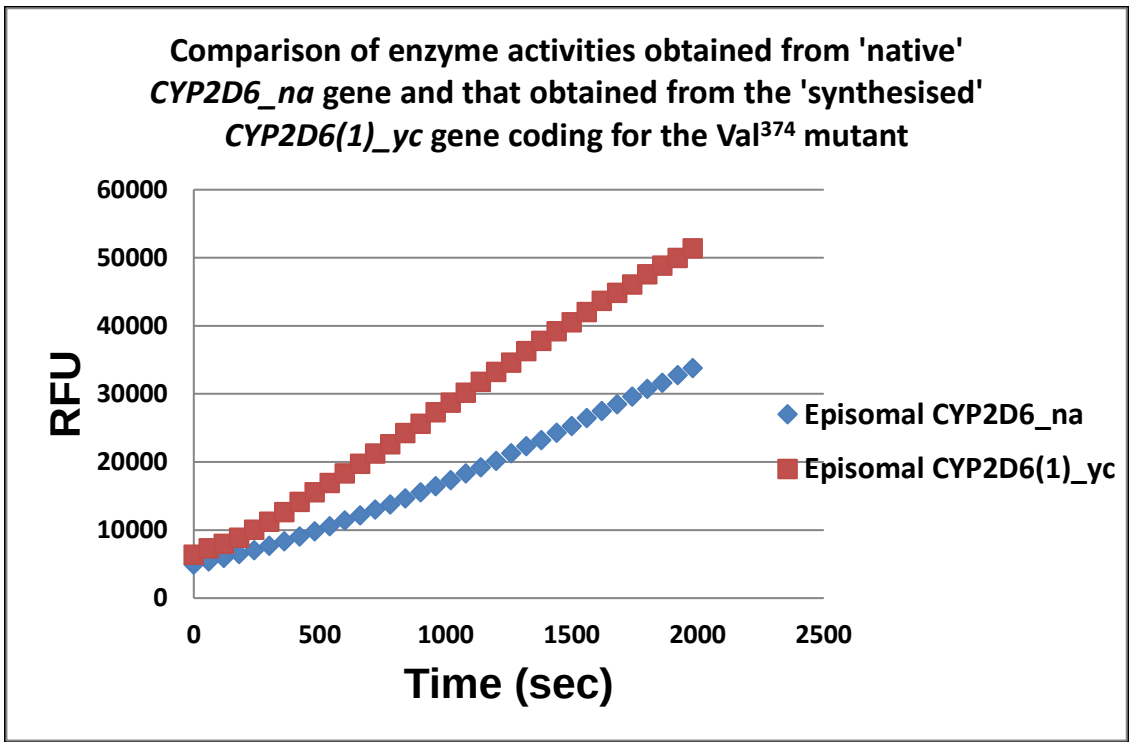


Figure 3.43. The graph compares the rate of reactions of (a) CYP2D6(1) enzyme expressed from a synthetic gene consisting of yeast biased codons, and (b) CYP2D6_na enzyme expressed from the native *CYP2D6* cDNA isolated from the human liver cDNA library. 7-ethoxymethoxy-3-cyanocoumarin (EOMCC) was used as substrate. EOMCC is converted to the fluorescent product 7-hydroxy-3-cyanocoumarin (HCC) upon reaction with CYP2D6. Rate was measured, over 30 min, in terms of relative fluorescence units (RFUs). The data represent mean ± S.D. of three independent experiments.

Both the 'synthesised' *CYP2D6(1)_yc* and the native *CYP2D6_na* genes were co-expressed only with the modified human *CPR* gene, *ΔhRDM*, but without any cytochrome b5. The results in Figure 3.43 show that the 'native' *CYP2D6_na* gene when expressed in yeast is produced less (blue) than the *CYP2D6* gene coding for the Val^{374} variant (Ton *et al*., 2004).

In the results from Figures 3.40 to 3.44, it has been assumed that the enzyme activities that have been measured directly correlate with the amounts of protein produced.

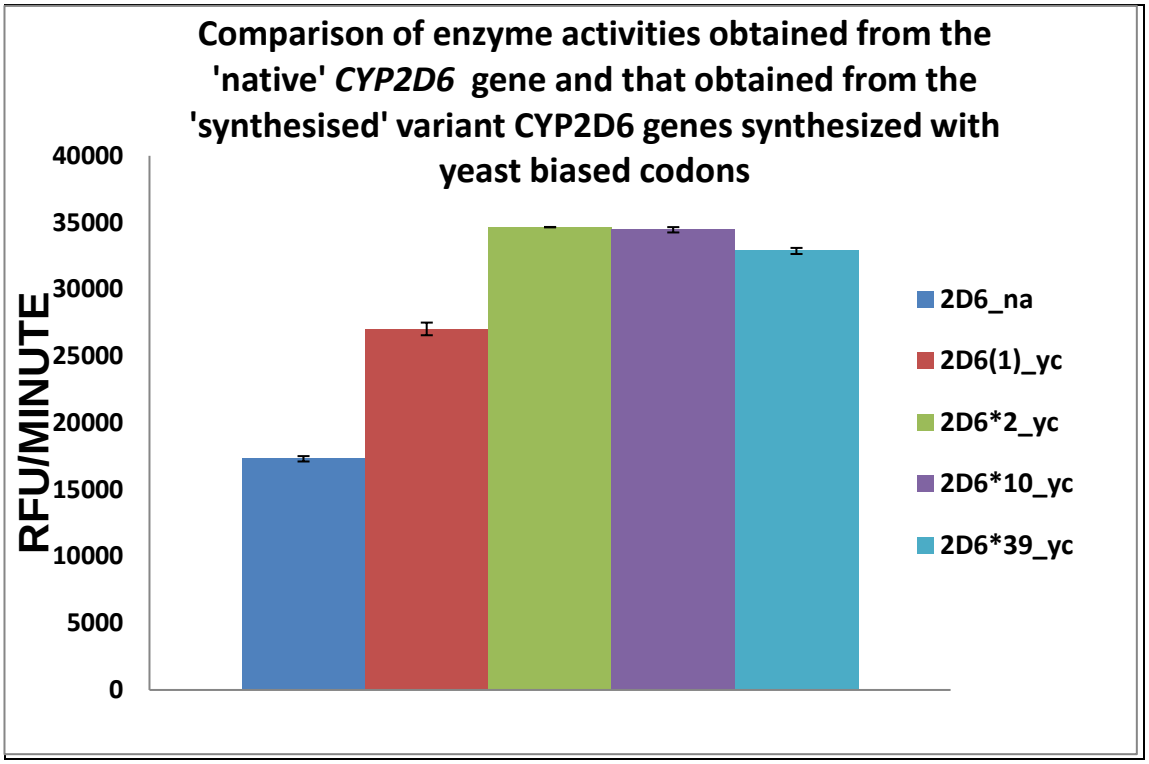


Figure 3.44. Comparison of activities of CYP2D6 enzyme, after 30 min of reaction obtained from (a) *CYP2D6_na*, (b) *CYP2D6(1)_yc*, (c) *CYP2D6*2_yc*, (d) *CYP2D6*10_yc*, and (e) *CYP2D6*39_yc* genes in strains derived from the yeast strain YY7. The bar plot compares the fluorescence emitted by 7-hydroxy-3-cyanocoumarin (HCC) after 30 min of reaction of CYP2D6 enzymes with 7-ethoxymethoxy-3-cyanocoumarin (EOMCC). The data represent mean ± S.D. of three independent experiments.

The results in Figure 3.44 would again indicate that there is more protein expressed from all the 'synthesized' *CYP2D6_yc* genes than from the 'native' *CYP2D6_na* gene because, as would be expected the gene with the yeast biased codons would provide more stable mRNA molecules (i.e. more stable mRNA relative to what would be transcribed from the 'native' gene) which would translate to more protein (relative to what would be produced by the 'native' mRNA). This was confirmed by performing Western blot analysis on

equal number of cells that express the variants ('synthesized' and 'native') of the *CYP2D6* gene.

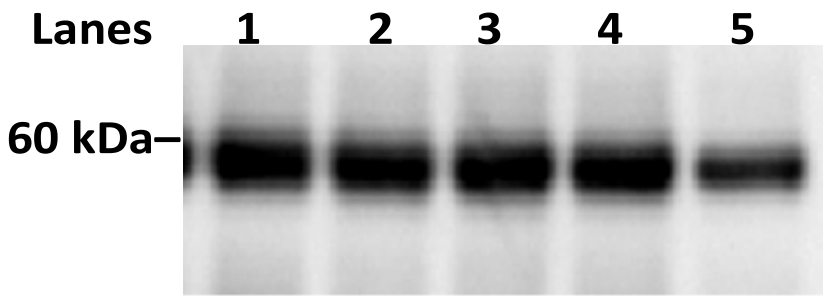


Figure 3.45. A Western blot which shows the CYP2D6 protein that is being expressed by equal number of cells (1X 10^6) from yeast strains that contain an episomal plasmid that bears 'chemically synthesized' genes *CYP2D6*10* (lane 1), *CYP2D6*2* (lane 2), *CYP2D6*39* (lane 3), *CYP2D6(1)* [i.e. CYP2D6(Val^{374}); lane 4], and 'native' *CYP2D6_na* (lane 5). The expected size of CYP2D6 protein is 55.8 kDa. The blot was probed by a human CYP2D6 specific monoclonal antibody (Santa Cruz Biotechnology, Cat no: sc130366).

The results shown in Figure 3.45 indicate that all the *CYP2D6_yc* genes produce similar amounts of CYP2D6 protein which is higher than that produced by the *CYP2D6_na* gene. Densitometric quantification (results not shown) corroborates the visual observations.

3.11 Cloning of *CYP2E1_yc* gene in the episomal plasmid pSY263 for expression of human CYP2E1 enzyme

3.11.1 Cloning of *CYP2E1_yc* gene in the episomal plasmid pSY263

CYP2E1 is another one of the CYP enzymes which is used for Drug Metabolism studies during the process of drug development (Zanger et al., 2013). The human *CYP2E1_yc* gene, with yeast biased codons, was cloned in the yeast expression vector pSYE263 using methodologies similar to cloning of the *CYP3A4_yc* gene in the same vector (this Chapter, Section 3.4.1). The resultant plasmid was named pSYE263/h_CYP2E1_yc.

A 1494 bp *BamH*I-*Xho*I *CYP2E1_yc* gene fragment (Figure 3.46) was isolated from the plasmid pUC57/BamHI-XhoI/h_CYP2E1_yc for DNA ligase mediated ligation to the 5711 bp *BamH*I-*Xho*I fragment of vector pSYE263, to create the episomal, 2µ-plasmid pSYE263/h_CYP2E1_yc (Figure 3.47) for expression of human CYP2E1 enzyme.

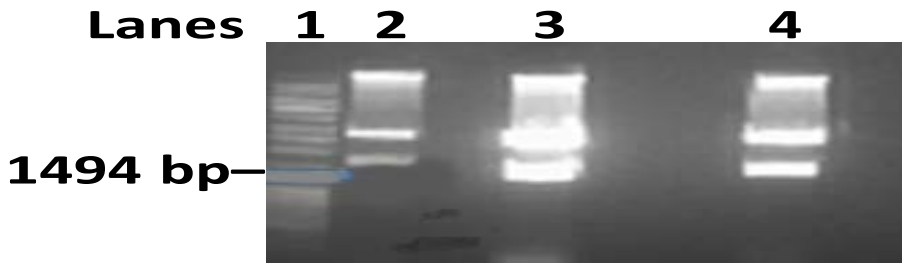


Figure 3.46. An agarose gel that shows the expected DNA fragments when plasmid pUC57/BamHI-Xhol/h_CYP2E1_yc was digested with enzymes *BamH*I and *Xho*l (lanes 2, 3, 4). The lower 1494 bp insert fragment (lanes 3 and 4) was ligated to the vector pSYE263 already digested with *BamH*I and *Xho*l to obtain the plasmid pSYE263/h_CYP2E1_yc. Lane 1, DNA ladder showing DNA bands of specific sizes; lane 2, uncut plasmid pUC57/BamHI-Xhol/h_CYP2E1_yc.

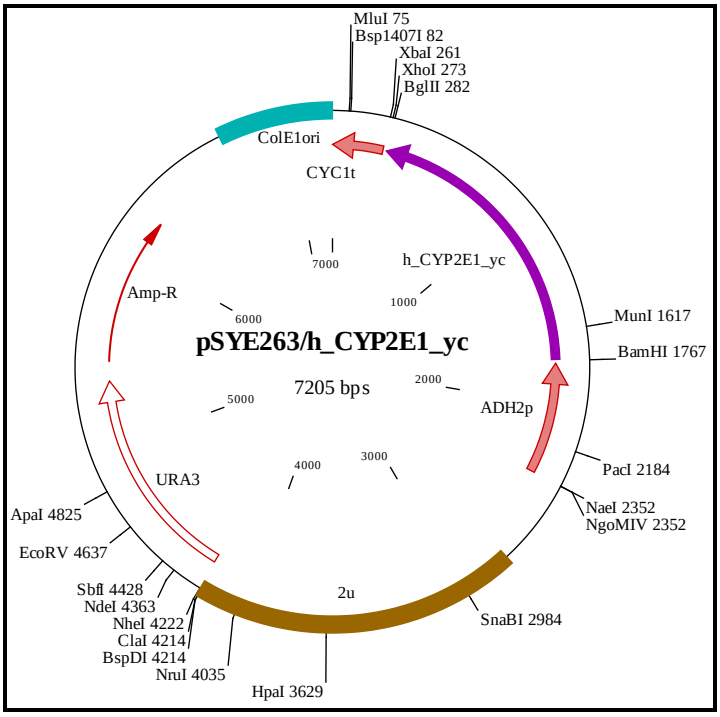


Figure 3.47. The map of the plasmid pSYE263/h_CYP2E1_yc, with restriction sites that occur in the plasmid only once. The *BamH*I and *Xho*I sites of pSYE263 were used for cloning the synthetic *h_CYP2E1_yc* gene with yeast biased codons, in the episomal 2µ-plasmid pSYE263.

After ligation and transformation of the ligation mixture in *E. coli* DH5α competent cells, individual bacterial transformants were grown for preparation of plasmid DNA using the alkaline lysis method (Chapter 2, Section 2.4.1.8).

The authenticity of the resultant plasmid DNA, pSYE263/h_CYP2E1_yc was confirmed by two separate digests with restriction enzymes (a) *BamH*I, *Xho*I and (b) *Pvu*II. It was verified that the newly constructed plasmid contained the correct size fragments (e.g. 1494 bp *BamH*I, *Xho*I insert; Figure 3.48).

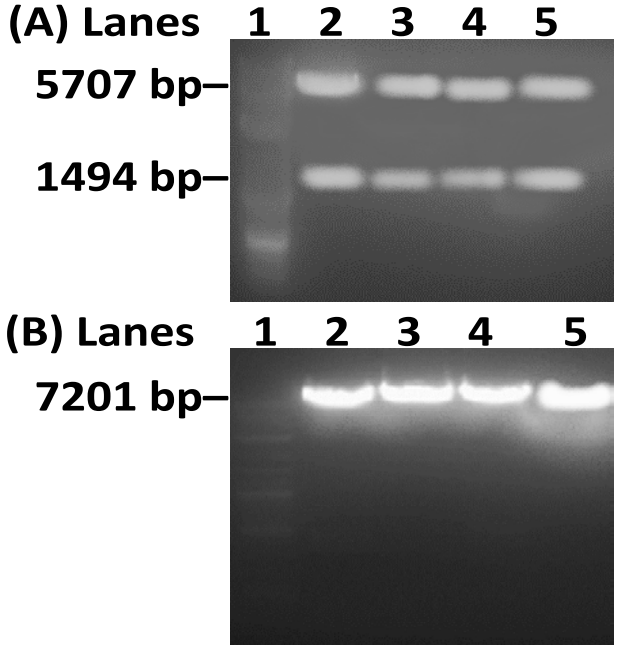


Figure 3.48. Agarose gels that show the expected DNA fragments when the plasmid pSYE263/h_CYP2E1_yc was digested with restriction enzymes (A) *BamH*I, *Xho*I and (B) *Pvu*II. Lane 1, DNA ladder; lanes 2 to 5, pSYE263/h_CYP1A2_yc digested with either *BamH*I-*Xho*I (A) or *Pvu*II (B).

3.11.2 Comparison of CYP2E1 enzyme activities expressed by 'synthesised' *CYP2E1_yc* and 'native' *CYP2E1_na* genes, both encoded by the episomal plasmid pSY263, using a fluorescence-based assay

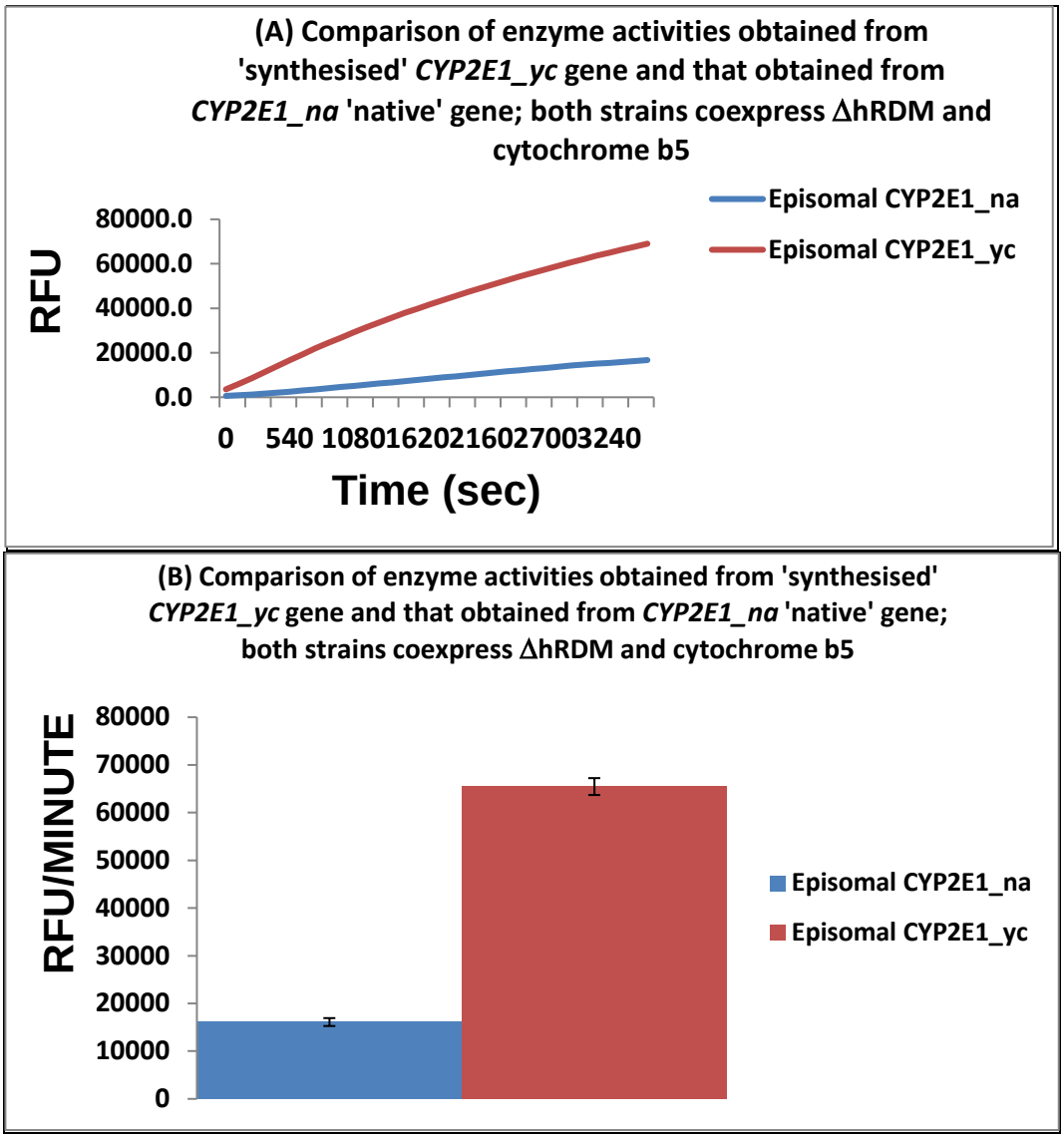


Figure 3.49. (A) The graphs show the rate at which the activity of the CYP2E1 enzyme, expressed in yeast strain YAB79 from the 'synthesised' *CYP2E1_yc* gene and the 'native' *CYP2E1_na* gene (co-expressed with *ΔhRDM* and cytochrome *b5* genes), increases over 30 min. Rate was measured in terms of relative fluorescence units (RFUs), using 7-ethoxymethoxy-3-cyanocumarin (EOMCC) as substrate. EOMCC is de-ethylated by CYP2E1 to form 7-hydroxy-3-cyanocoumarin. (B) Depicts the comparison of fluorescence emitted by the two yeast strains at 30 min of reaction of enzyme with substrate. The data represent mean ± S.D. of three independent experiments.

The results shown in Figure 3.49 (above) indicate that the 'synthesised' *CYP2E1_yc* gene produces more CYP2E1 enzyme than the 'native' *CYP2E1_na* gene. Once again the results prove that the yeast biased codons are providing more mRNA because it is more stable than the mRNA transcribed by the 'native'. Hence, *CYP2E1_yc* gene is producing more protein.

That the mRNA derived from yeast biased codons *CYP2E1* coding sequence translates to more protein, relative to what is produced by the 'native' mRNA, was confirmed by Western blotting, using equal number of cells (1X 10^6), that express the two variant *CYP2E1* genes, 'synthesised' and 'native' (Figure 3.50). Densitometric quantification (results not shown) suggests that ~4 times more protein is produced by *CYP2E1_yc* gene than by the native *CYP2E1_na* gene isolated from a human liver cDNA library.

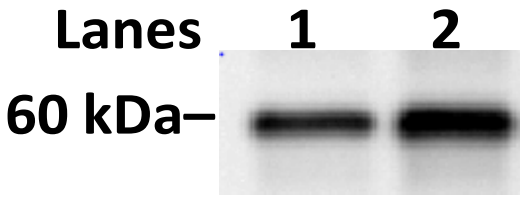


Figure 3.50. Western blot that shows the amounts of CYP2E1 protein being expressed by equal number of cells (1X 10^6) from different yeast strains. Lane 1: protein from cells which contain the plasmid pSYE263/CYP2E1_na (lane 1); lane 2: protein from cells which contain the plasmid pSYE263/CYP2E1_yc (lane 2). The expected size of CYP2E1 protein is 56.9 kDa. The blot was probed by a human CYP2E1 specific monoclonal antibody (Santa Cruz Biotechnology, Cat no: sc133491).

3.12 Cloning of *CYP2A6_yc* gene in the episomal plasmid pSY263 for expression of human CYP2A6 enzyme

3.12.1 Cloning of *CYP2A6_yc* gene in the episomal plasmid pSY263

CYP2A6 is the ninth CYP enzyme which has been cloned during the work conducted for this thesis. As pointed out earlier there are ten to twelve CYP enzymes which are commonly used for Drug Metabolism studies in the process of drug development, CYP2A6 being one of them (Zanger et al., 2013). The human *CYP2A6_yc* gene, with yeast biased codons, was cloned in the yeast expression vector pSYE263 using methodologies similar to cloning of the *CYP3A4_yc* gene in the same vector (this Chapter, Section 3.4.1). The resultant plasmid was named pSYE263/h_CYP2A6_yc.

A 1497 bp *BamH*I-*Xho*I *CYP2A6_yc* gene fragment (Figure 3.51) was isolated from the plasmid pUC57/BamHI-XhoI/h_CYP2A6_yc for DNA ligase mediated ligation to the 5711 bp *BamH*I-*Xho*I fragment of vector pSYE263, to create the episomal, 2µ-plasmid pSYE263/h_CYP2A6_yc (Figure 3.52) for expression of human CYP2A6 enzyme.

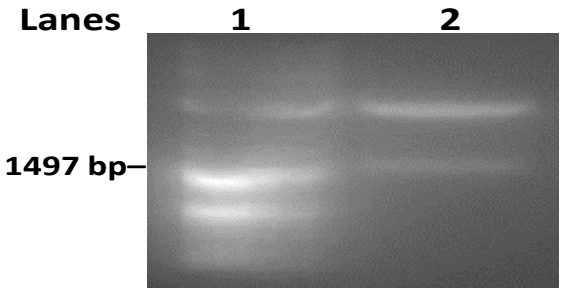


Figure 3.51. An agarose gel that shows the expected DNA fragments when the plasmid pUC57/BamHI-Xhol/h_CYP2A6_yc was digested with *BamH*I and *Xho*l (lane 2). The 1497 bp insert fragment was isolated from the gel and ligated to the pSYE263 vector which had also been digested with *BamH*I and *Xho*l to obtain the plasmid pSYE263/h_CYP2A6_yc. Lane 1, DNA ladder with DNA fragments with defined base pairs.

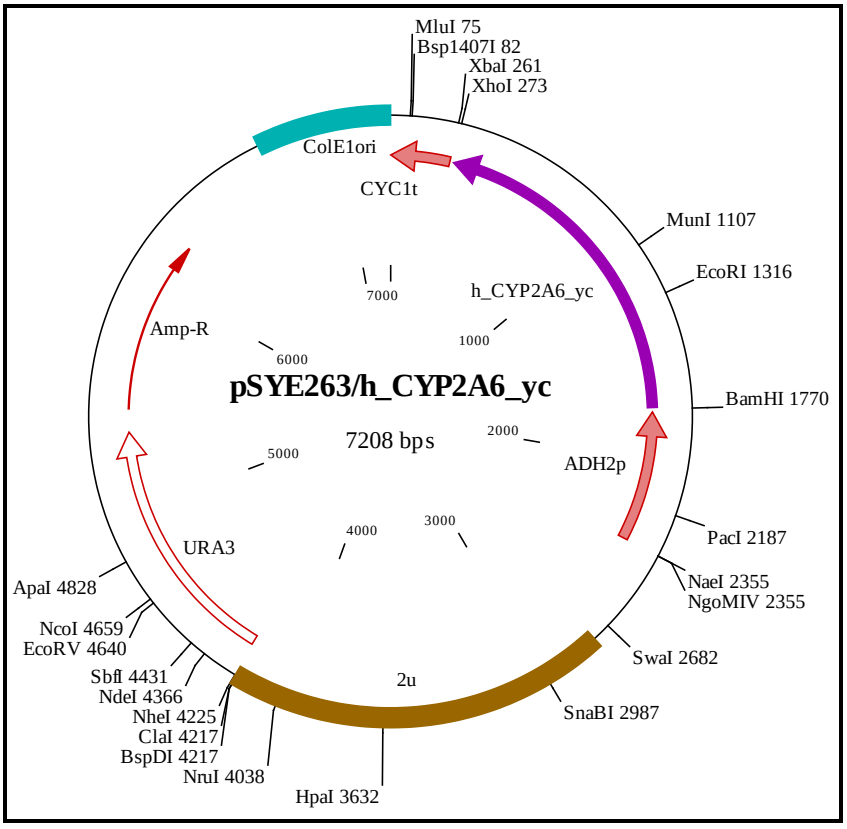


Figure 3.52. The map of the plasmid pSYE263/h_CYP2A6_yc, with restriction sites that occur in the plasmid only once. The *BamH*I and *Xho*I sites of pSYE263 were used for cloning the synthetic *h_CYP2A6_yc* gene with yeast biased codons, in the episomal 2µ-plasmid pSYE263.

After ligation and transformation of the ligation mixture in *E. coli* DH5α competent cells, individual bacterial transformants were grown for preparation of plasmid DNA using the alkaline lysis method (Chapter 2, Section 2.4.1.8).

The authenticity of the resultant plasmid DNA, pSYE263/h_CYP2A6_yc was confirmed by two separate digests with restriction enzymes (a) *BamH*I, *Xho*I and (b) *BamH*I, *Xba*I. It was verified that the newly constructed plasmid contained the correct size fragments (e.g. 1497 bp *BamH*I, *Xho*I insert; Figure 3.53).

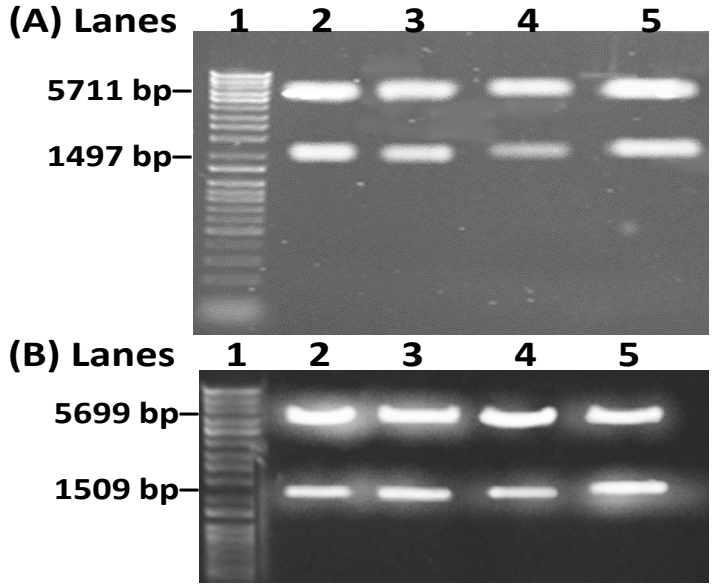


Figure 3.53. Agarose gels that show the expected DNA fragments when the plasmid pSYE263/h_CYP2A6_yc was digested with restriction enzymes (A) *BamH*I, *Xho*I and (B) *BamH*I, *Xba*I. Lane 1, DNA ladder; lanes 2 to 5, pSYE263/h_CYP2B6_yc digested with either *BamH*I-*Xho*I (A) or *BamH*I-*Xba*I (B).

3.12.2 Comparison of CYP2A6 enzyme activities expressed by 'synthesised' *CYP2A6_yc* and 'native' *CYP2A6_na* genes, both encoded by the episomal plasmid pSY263, using a fluorescence-based assay

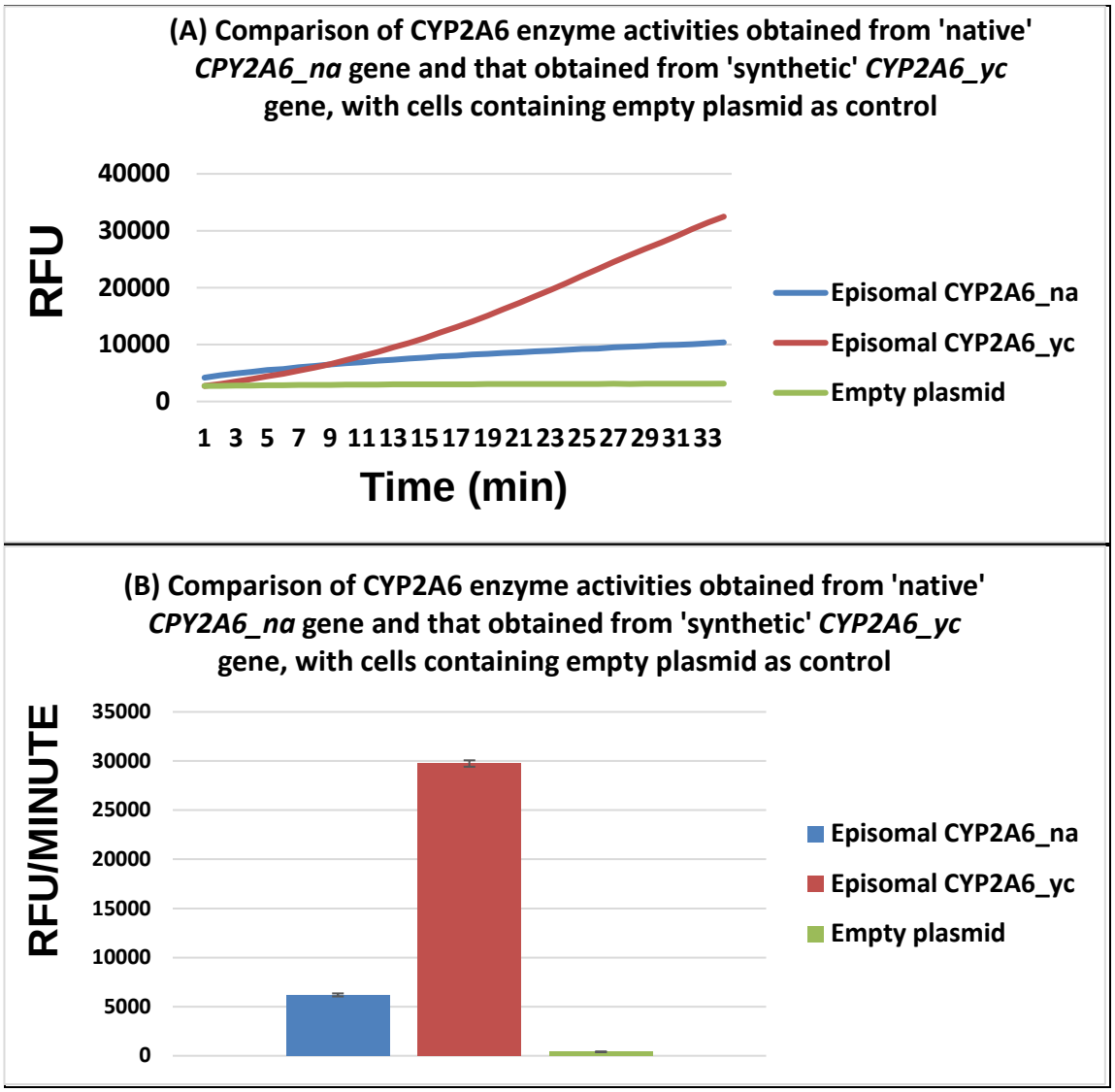


Figure 3.54. (A) The graphs show the rate at which the activity of the CYP2A6 enzyme, expressed in yeast strain YAB79 from the 'synthesised' *CYP2A6_yc* gene and the 'native' *CYP2A6_na* gene (co-expressed with *ΔhRDM* and cytochrome *b5* genes), increases over 30 min. Rate was measured in terms of relative fluorescence units (RFUs) using 7-methoxy-3-cyanocoumarin (CMC) as substrate. CMC is de-ethylated by CYP2A6 to form 7-hydroxy-3-cyanocoumarin (HCC). (B) Depicts the comparison of fluorescence emitted by the three yeast strains at 30 min of reaction of enzyme with substrate. The data represent mean ± S.D. of three independent experiments.

The results shown in Figure 3.54 (above) indicate that the 'synthesised' *CYP2A6_yc* gene produces much more CYP2A6 enzyme than the 'native' *CYP2A6_na* gene. Since the gene with the yeast biased codons should provide mRNA more stable than the mRNA transcribed by the 'native' gene, production of more protein from the synthetic gene was expected.

The mRNA derived from yeast biased codons *CYP2A6* coding sequence should translate to more protein relative to what would be produced by the 'native' mRNA. This was confirmed by Western blotting, using equal number of cells (1X 10^6), that express the two variants, 'synthesised' and 'native', of the *CYP2A6* gene (Figure 3.55). Densitometric quantification (results not shown) suggests that at least 10 times more protein is produced by *CYP2A6_yc* gene than by the native *CYP2A6_na* gene isolated from a human liver cDNA library.

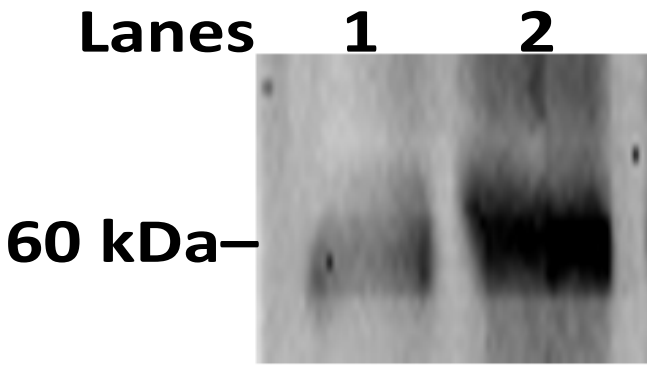


Figure 3.55. Western blot that shows the amounts of CYP2A6 protein being expressed by equal number of cells (1X 10^6) from different yeast strains. Lane 1: protein from cells which contain the plasmid pSYE263/CYP2A6_na (lane 1); lane 2: protein from cells which contain the plasmid pSYE263/CYP2A6_yc (lane 2). The expected size of CYP2A6 protein is 56.5 kDa. The blot was probed by a human CYP2A6 specific monoclonal antibody (Santa Cruz Biotechnology, Cat no: sc53615).

3.13 Cloning of *CYP2C8_yc* gene in the episomal plasmid pSY263 for expression of human CYP2C8 enzyme

3.13.1 Cloning of *CYP2C8_yc* gene in the episomal plasmid pSY263

CYP2C8 is the tenth CYP enzyme which was cloned during the work conducted for this thesis. As pointed out earlier there are ten main CYP enzymes which are commonly used for Drug Metabolism studies in the process of drug development. This is the tenth enzyme in that list of enzymes ((Zhang et al., 2013)). The human *CYP2C8_yc* gene, with yeast biased codons, was cloned in the yeast expression vector pSYE263 using methodologies similar to cloning of the *CYP2C8_yc* gene in the same vector (this Chapter, Section 3.4.1). The resultant plasmid was named pSYE263/h_CYP2C8_yc.

A 1485 bp *BamH*I-*Xho*I *CYP2C8_yc* gene fragment (Figure 3.56) was isolated from the plasmid pUC57/BamHI-XhoI/h_CYP2C8_yc for DNA ligase mediated ligation to the 5711 bp *BamH*I-*Xho*I fragment of vector pSYE263, to create the episomal, 2µ-plasmid pSYE263/h_CYP2C8_yc (Figure 3.57) for expression of human CYP2C8 enzyme.

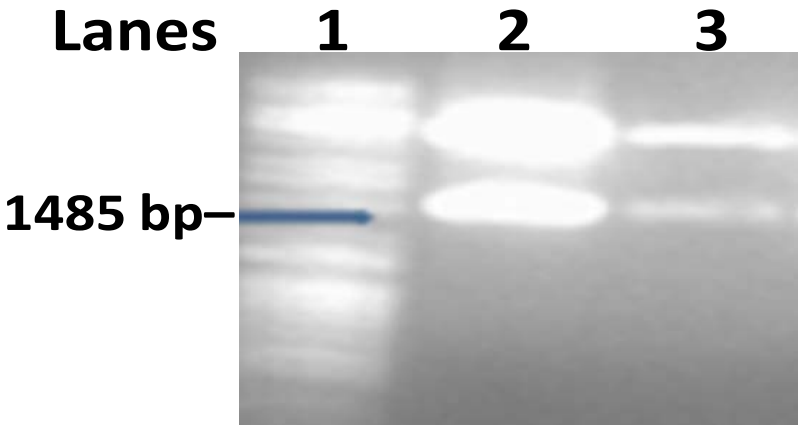


Figure 3.56. An agarose gel that shows the expected DNA fragments when the plasmid pUC57/BamHI-Xhol/h_CYPC8_yc was digested with *BamH*I and *Xho*l. The 1485 bp insert fragment was isolated from the gel and ligated to the pSYE263 vector which had also been digested with *BamH*I and *Xho*l to obtain the plasmid pSYE263/h_CYPC8_yc.

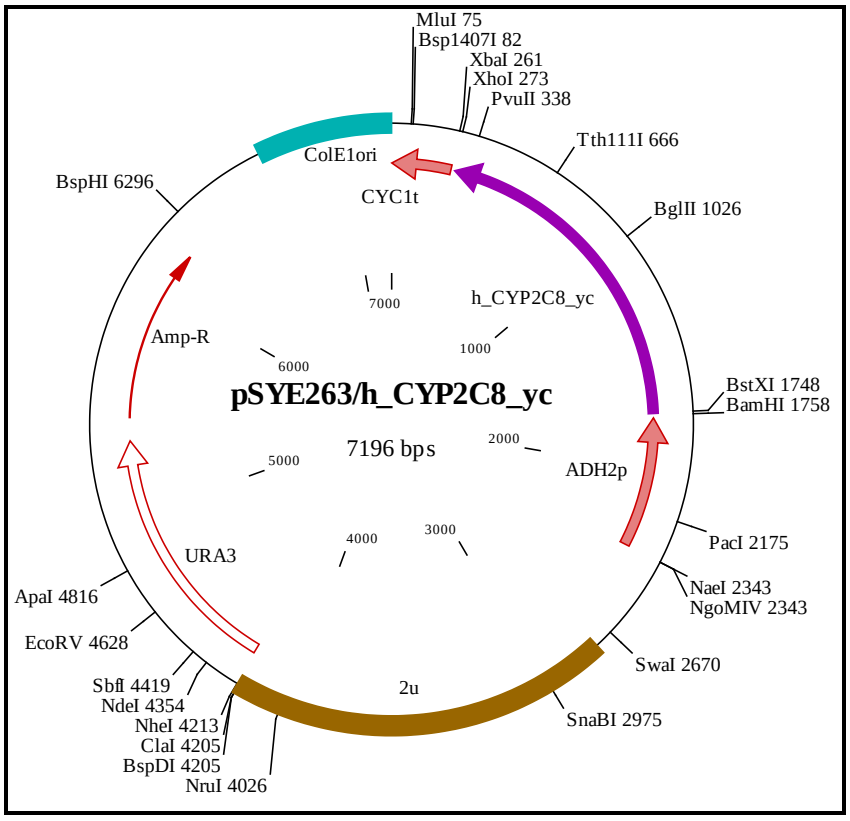


Figure 3.57. The map of the plasmid pSYE263/h_CYP2C8_yc, with restriction sites that occur in the plasmid only once. The *BamH*I and *Xho*I sites of pSYE263 were used for cloning the synthetic *h_CYP2C8_yc* gene with yeast biased codons, in the episomal 2µ-plasmid pSYE263.

After ligation and transformation of the ligation mixture in *E. coli* DH5α competent cells, individual bacterial transformants were grown for preparation of plasmid DNA using the alkaline lysis method (Chapter 2, Section 2.4.1.8).

The authenticity of the resultant plasmid DNA, pSYE263/h_CYP2C8_yc was confirmed by digestion with restriction enzymes *BamH*I, *Xho*I. It was verified that the newly constructed plasmid contained the correct size gene insert fragment (e.g. 1485 bp *BamH*I, *Xho*I insert; Figure 3.58).

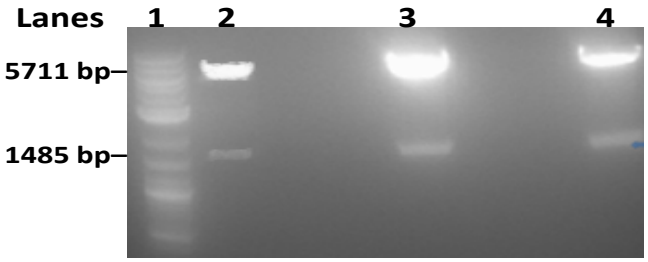


Figure 3.58. An agarose gel that shows the expected DNA fragments when the plasmid pSYE263/BamHI-Xhol/h_CYP2C8_yc was digested with *BamH*I and *Xho*I restriction enzymes (lanes 2, 3, 4). The 1485 bp gene insert fragment was isolated from the gel and ligated with the pSYE263 vector which had been digested with *BamH*I and *Xho*I to obtain the plasmid pSYE263/ h_CYP2C8_yc. Lane 1, DNA ladder showing DNA fragments with defined base pairs.

3.13.2 Comparison of CYP2C8 enzyme activities expressed by 'synthesised' *CYP2C8_yc* and 'native' *CYP2C8_na* genes, both encoded by the episomal plasmid pSY263, using a fluorescence-based assay

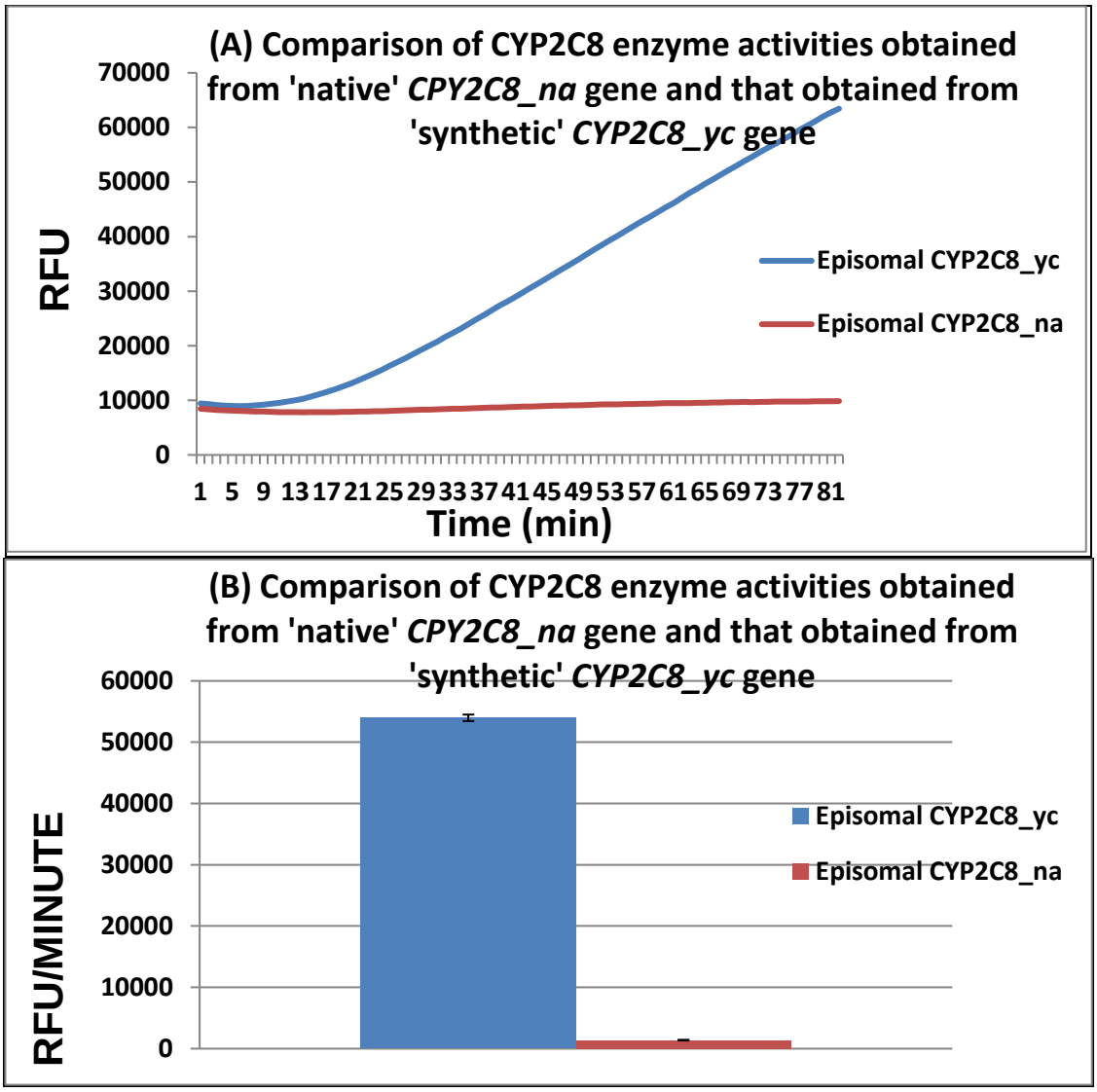


Figure 3.59. (A) The graphs show the rate at which the activity of the CYP2C8 enzyme, expressed in yeast strain YAB79 from the 'synthesised' *CYP2C8_yc* gene and the 'native' *CYP2C8_na* gene (co-expressed with *ΔhRDM* and cytochrome *b5* genes), increases over 30 min. Rate was measured in terms of relative fluorescence units (RFUs) using 7-ethoxymethoxy-3-cyanocoumarin (EOMCC) was used as substrate. EOMCC is de-ethylated to the fluorescent product 7-hydroxy-3-cyanocoumarin (HCC) upon reaction with CYP2C8. (B) Depicts the comparison of fluorescence emitted by the two yeast strains at 30 min of reaction of enzyme with substrate. The data represent mean ± S.D. of three independent experiments.

The results shown in Figure 3.59 (above) indicate that the 'synthesised' *CYP2C8_yc* gene produces a lot more CYP2C8 enzyme than the 'native' *CYP2C8_na* gene in YAB79 derived strains. Since the gene with the yeast biased codons should provide mRNA more stable than the mRNA transcribed by the 'native' gene, production of more protein from the synthetic gene was expected.

The mRNA derived from yeast biased codons *CYP2C8* coding sequence should translate to more protein relative to what would be produced by the 'native' mRNA. This was confirmed by Western blotting, using equal number of cells (1X 10^6), that express the two variants, 'synthesised' and 'native', of the *CYP2C8* gene (Figure 3.60). Densitometric quantification (results not shown) suggests that at least 6 times more protein is produced by *CYP2C8_yc* gene than by the native *CYP2C8_na* gene isolated from a human liver cDNA library.

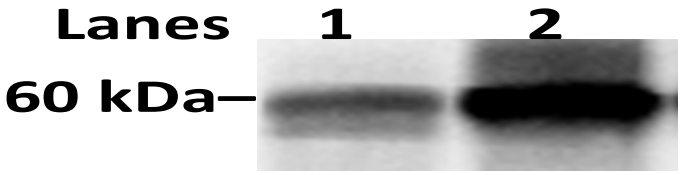


Figure 3.60. Western blot that shows the amounts of CYP2C8 protein being expressed by equal number of cells (1X 10^6) from different yeast strains. Lane 1: protein from cells which contain the plasmid pSYE263/CYP2C8_na (lane 1); lane 2: protein from cells which contain the plasmid pSYE263/CYP2C8_yc (lane 2). The expected size of CYP2C8 protein is 55.8 kDa. The blot was probed by a human CYP2C8 specific monoclonal antibody (Santa Cruz Biotechnology, Cat no: sc154136).

3.14 Cloning of *CYP1A1_yc* gene in the episomal plasmid pSY263 for expression of human CYP1A1 enzyme

3.14.1 Cloning of *CYP1A1_yc* gene in the episomal plasmid pSY263

CYP1A1 is another CYP enzyme whose gene was cloned during the work conducted for this thesis. Only recently it has been pointed out that CYP1A1 has a role in drug metabolism. Moreover is a target for finding anticancer agents that can act as chemo-preventives. Some new chemical scaffolds have been identified by our group (where I was part of the discovery) using CYP1A1 microsomal enzyme produced in yeast (Mohd Siddique et al., 2016). This is the eleventh enzyme in that list of enzymes that was cloned for the work described in this thesis.

The human *CYP1A1_yc* gene, with yeast biased codons, was cloned in the yeast expression vector pSYE263 using methodologies similar to cloning of the *CYP3A4_yc* gene in the same vector (this Chapter, Section 3.4.1). The resultant plasmid was named pSYE263/h_CYP1A1_yc.

A 1551 bp *BamH*I-*Xho*I *CYP1A1_yc* gene fragment (Figure 3.61) was isolated from the plasmid pUC57/BamHI-XhoI/h_CYP1A1_yc for DNA ligase mediated ligation to the 5711 bp *BamH*I-*Xho*I fragment of vector pSYE263, to create the episomal, 2µ-plasmid pSYE263/h_CYP1A1_yc (Figure 3.62) for expression of human CYP1A1 enzyme.

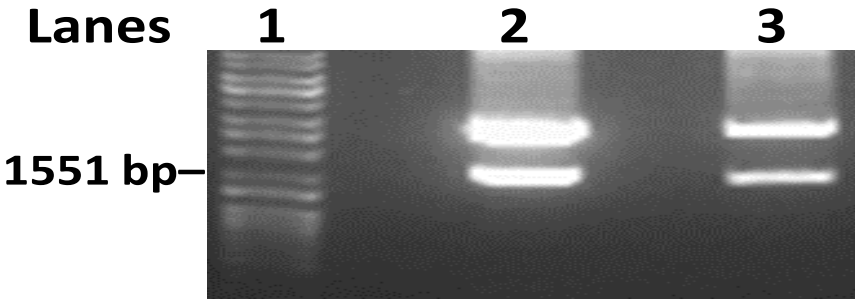


Figure 3.61. An agarose gel that shows the expected DNA fragments when the plasmid pUC57/BamHI-XhoI/h_CYP1A1_yc was digested with *BamH*I and *Xho*I (lanes 2, 3). The 1551 bp gene insert fragment was isolated from the gel and ligated to the vector pSYE263 already digested with *BamH*I and *Xho*I to obtain the plasmid pSYE263/h_CYP1A1_yc. Lane 1, DNA ladder with DNA fragments with defined base pairs.

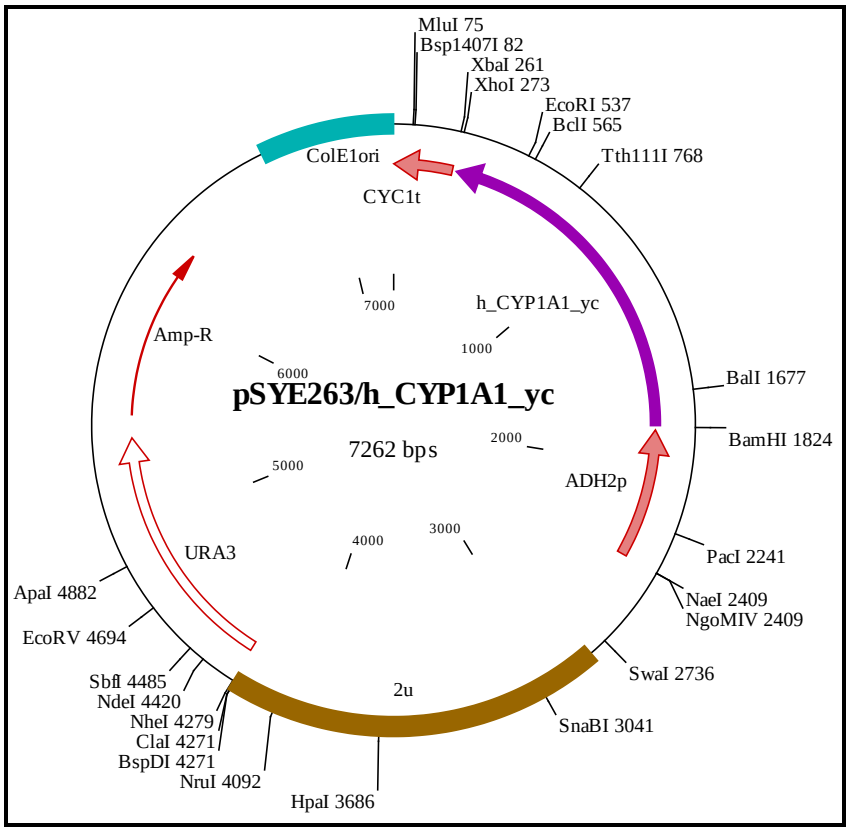


Figure 3.62. The map of the plasmid pSYE263/h_CYP1A1_yc, with restriction sites that occur in the plasmid only once. The *BamH*I and *Xho*I sites of pSYE263 were used for cloning the synthetic *h_CYP1A1_yc* gene with yeast biased codons, in the episomal 2μ-plasmid pSYE263.

After ligation and transformation of the ligation mixture in *E. coli* DH5α competent cells, individual bacterial transformants were grown for preparation of plasmid DNA using the alkaline lysis method (Chapter 2, Section 2.4.1.8).

The authenticity of the resultant plasmid DNA, pSYE263/h_CYP1A1_yc was confirmed by two separate digests with restriction enzymes (a) *BamH*I, *Xho*I and (b) *BamH*I, *Xba*I. It was verified that the newly constructed plasmid contained the correct size fragments (e.g. 1551 bp *BamH*I, *Xho*I insert; Figure 3.63).

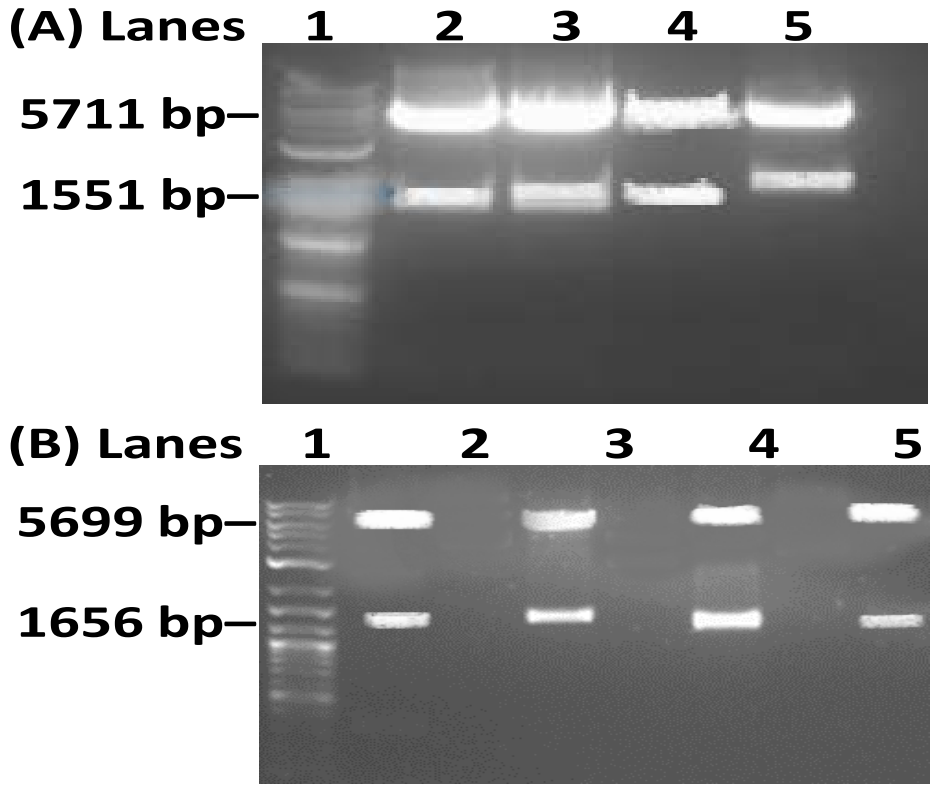


Figure 3.63. Agarose gels that show the expected DNA fragments when the plasmid pSYE263/h_CYP1A1_yc was digested with restriction enzymes (A) *BamH*I, *Xho*I and (B) *BamH*I, *Xba*I. Lane 1, DNA ladder; lanes 2 to 5, pSYE263/h_CYP1A1_yc digested with either *BamH*I-*Xho*I (A) or *BamH*I-*Xba*I (B).

3.14.2 Comparison of CYP1A1 enzyme activities expressed by 'synthesised' *CYP1A1_yc* and 'native' *CYP1A1_na* genes, both encoded by the episomal plasmid pSY263, using a fluorescence-based assay

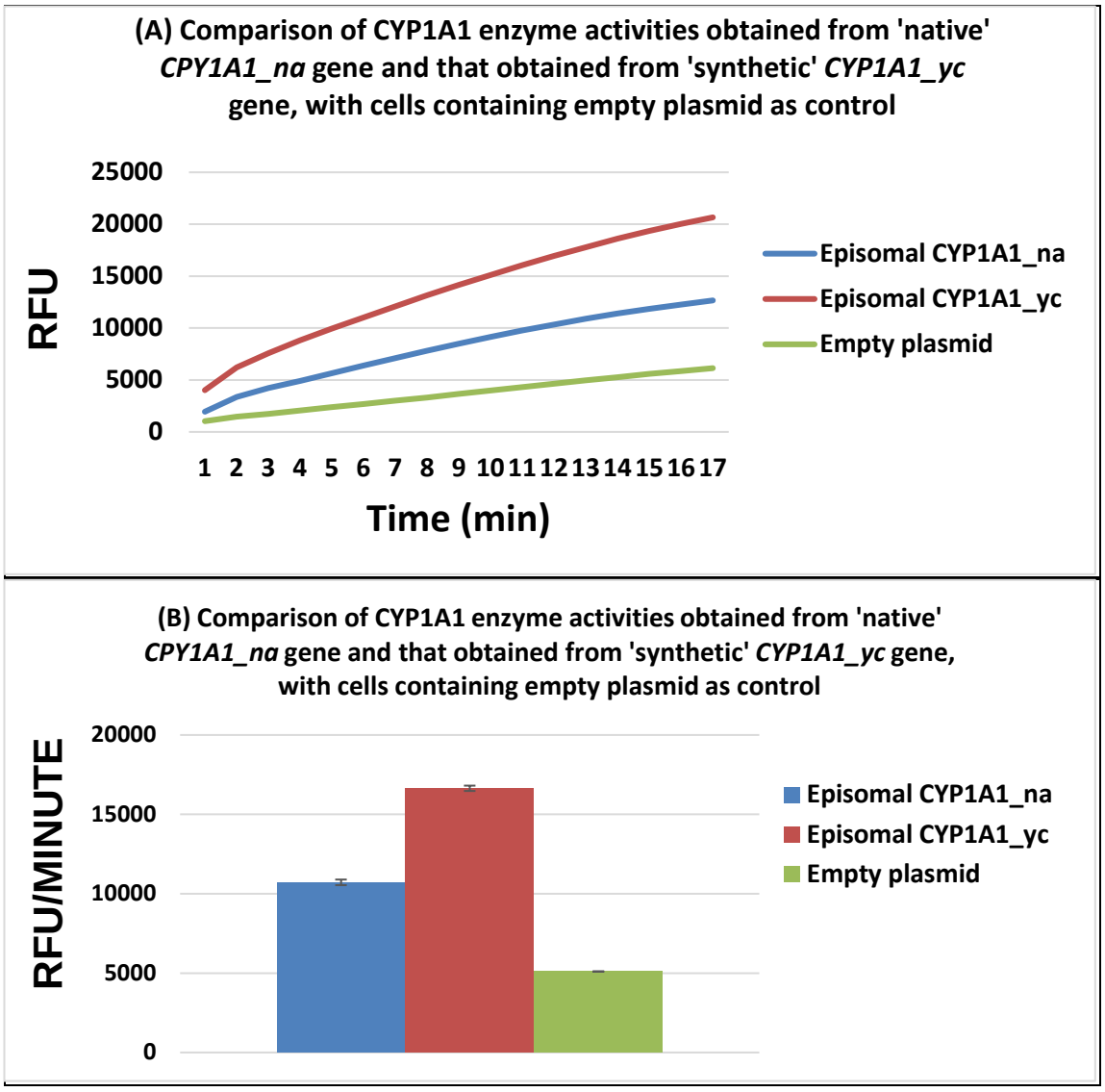


Figure 3.64. (A) The graphs show the rate at which the activity of the CYP1A1 enzyme, expressed in yeast strain YY7 from the 'synthesised' *CYP1A1_yc* gene and the 'native' *CYP1A1_na* gene (co-expressed with only *ΔhRDM* gene), increases over 16 min. Rate was measured in terms of relative fluorescence units (RFUs) using 7-ethoxy resorufin (7-ER) as substrate. 7-ER is de-ethylated by CYP1A1 to form resorufin. (B) Depicts the comparison of fluorescence emitted by the three yeast strains at 16 min of reaction of enzyme with substrate. The data represent mean ± S.D. of three independent experiments.

The results shown in Figure 3.64 (above) indicate that the 'synthesised' *CYP1A1_yc* gene produces much more CYP1A1 enzyme than the 'native' *CYP1A1_na* gene in YY7 derived yeast strains. Since the gene with the yeast biased codons should provide mRNA more stable than the mRNA transcribed by the 'native' gene, production of more protein from the synthetic gene was expected.

The mRNA derived from yeast biased codons *CYP1A1* coding sequence again did transcribe/translate to more protein relative to what was produced by the 'native' mRNA. This was confirmed by Western blotting, using equal number of cells (1X 10^6), that express the two variants, 'synthesised' and 'native', of the *CYP1A1* gene (Figure 3.65). Densitometric quantification (results not shown) suggests that ~2 times more protein is produced by *CYP1A1_yc* gene than by the native *CYP1A1_na* gene isolated from a human liver cDNA library.

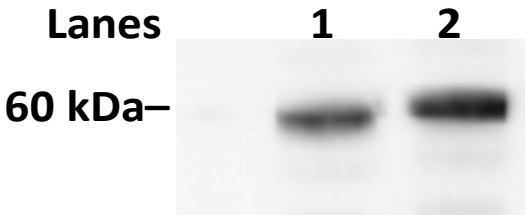


Figure 3.65. Western blot that shows the amounts of CYP1A1 protein being expressed by equal number of cells (1X 10^6) from different yeast strains. Lane 1: protein from cells which contain the plasmid pSYE263/CYP1A1_na (lane 1); lane 2: protein from cells which contain the plasmid pSYE263/CYP1A1_yc (lane 2). The expected size of CYP1A1 protein is 58.2 kDa. The blot was probed by a human CYP1A1 specific monoclonal antibody (Santa Cruz Biotechnology, Cat no: sc393979).

3.15 Cloning of *CYP1B1_yc* gene in the episomal plasmid pSY263 for expression of human CYP1B1 enzyme

3.15.1 Cloning of *CYP1B1_yc* gene in the episomal plasmid pSY263

Like CYP1A1, CYP1B1 is considered a target for finding novel anticancer agents. It has been suggested that CYP1B1 inhibitors can have a role in the prevention of hormone dependent cancers related to the breast and prostate (Mohd et al., 2016; a publication from our group). The human *CYP1B1* gene was cloned for expression in yeast. Some interesting chemical scaffolds have been identified by our group using CYP1B1 microsomal enzyme produced in yeast (Horley et al., 2017; another publication from our group). This is the twelfth enzyme in the list of enzymes that was cloned for the work described in this thesis.

The human *CYP1B1_yc* gene, with yeast biased codons, was cloned in the yeast expression vector pSYE263 using methodologies similar to cloning of the *CYP3A4_yc* gene in the same pSYE263 vector (this Chapter, Section 3.4.1). The resultant plasmid was named pSYE263/h_CYP1B1_yc.

A 1563 bp *BamH*I-*Xho*I *CYP1B1_yc* gene fragment (Figure 3.66) was isolated from the plasmid pUC57/BamHI-XhoI/h_CYP1B1_yc for DNA ligase mediated ligation to the 5711 bp *BamH*I-*Xho*I fragment of vector pSYE263, to create the episomal, 2µ-plasmid pSYE263/h_CYP1B1_yc (Figure 3.67) for expression of human CYP1B1 enzyme.

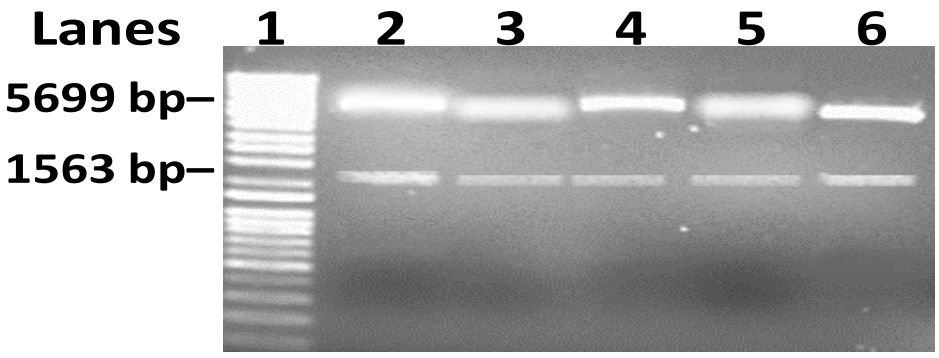


Figure 3.66. An agarose gel that shows the expected DNA fragments when the plasmid pSYE263/BamHI-XhoI/h_CYP1B1_yc was digested with BamHI and XhoI (lanes 2 to 6). The 1563 bp gene insert fragment was isolated from the gel and ligated with pSYE263 vector digested with BamHI, XhoI to obtain the plasmid pSYE263/h_CYP1B1_yc. Lane 1, DNA ladder showing DNA fragments with defined base pairs.

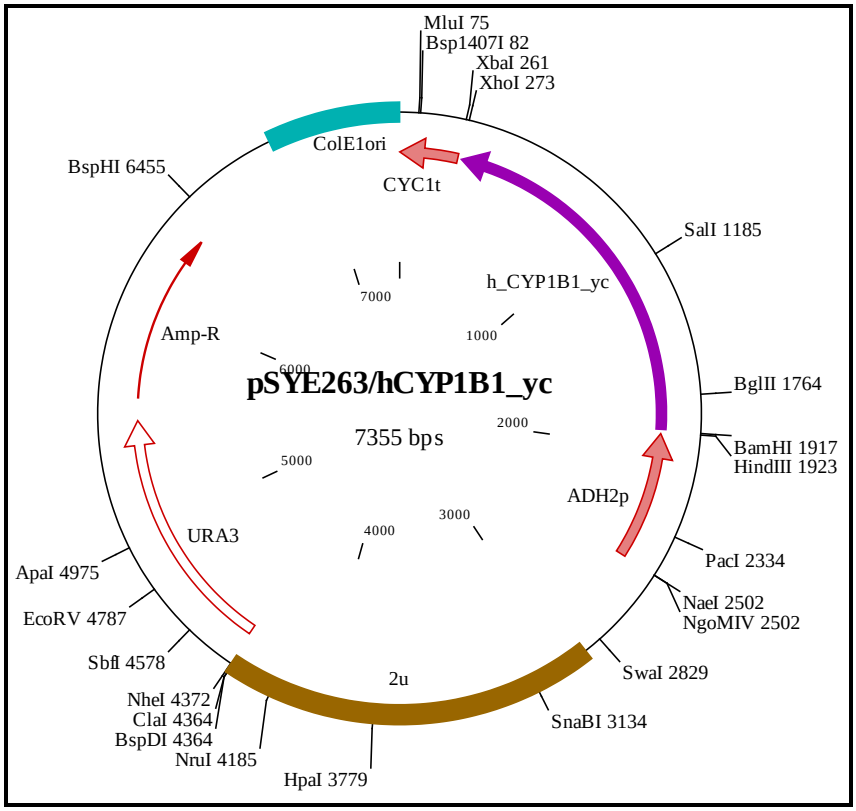


Figure 3.67. The map of the plasmid pSYE263/h_CYP1B1_yc, with restriction sites that occur in the plasmid only once. The *BamH*I and *Xho*I sites of pSYE263 were used for cloning the synthetic *h_CYP1B1_yc* gene with yeast biased codons, in the episomal 2µ-plasmid pSYE263.

After ligation and transformation of the ligation mixture in *E. coli* DH5α competent cells, individual bacterial transformants were grown for preparation of plasmid DNA using the alkaline lysis method (Chapter 2, Section 2.4.1.8).

The authenticity of the resultant plasmid DNA, pSYE263/h_CYP1B1_yc was confirmed by digestion with restriction enzymes *BamH*I, *Xba*I. It was verified that the newly constructed plasmid contained the correct size fragments (Figure 3.68).

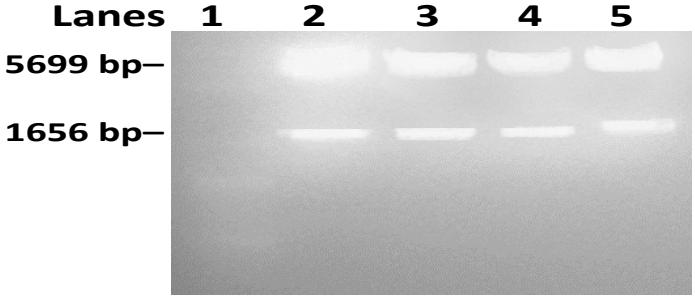


Figure 3.68. An agarose gel that shows the expected DNA fragments when the plasmid pSYE263/BamHI-Xhol/h_CYP1B1_yc was digested with *BamH*I and *Xba*I restriction enzymes (lanes 2 to 5). Lane 1, DNA ladder.

3.15.2 Comparison of CYP1B1 enzyme activities expressed by 'synthesised' *CYP1B1_yc* and 'native' *CYP1B1_na* genes, both encoded by the episomal plasmid pSY263, using a fluorescence-based assay

The results shown in Figure 3.69 (below) indicate that the 'synthesised' *CYP1B1_yc* gene produces a lot more CYP1B1 active enzyme than the 'native' *CYP1B1_na* gene in YY7 (containing only the modified human CPR, *ΔhRDM*, gene) derived yeast strains. In the case of CYP1B1, the yeast biased codons do provide a far more stable mRNA than the mRNA transcribed by the 'native' gene. This should also be reflected in the different levels of production of CYP1B1 protein from the two variant genes.

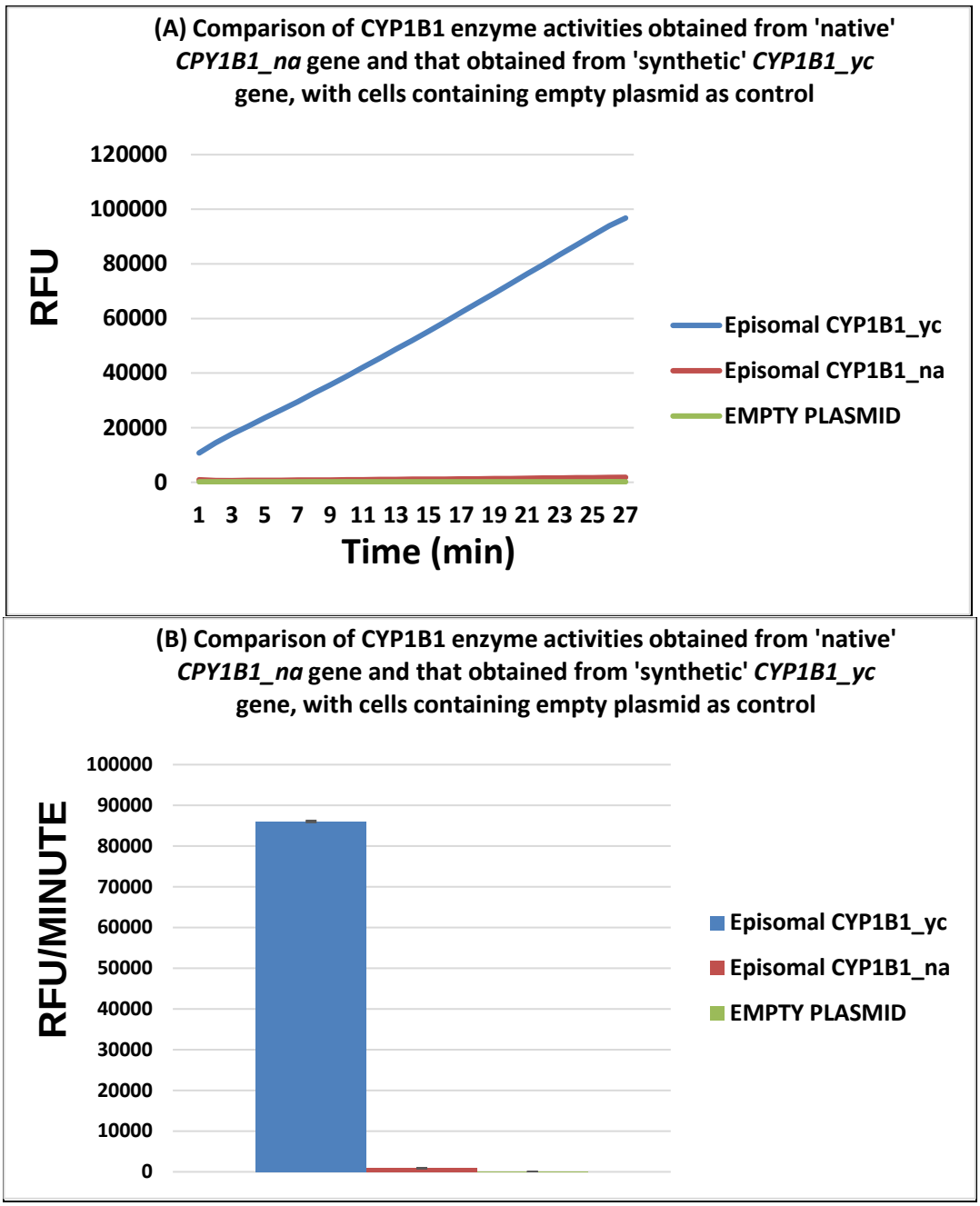


Figure 3.69. (A) The graphs show the rate at which the activity of the CYP1B1 enzyme, expressed in yeast strain YY7 from the 'synthesised' *CYP1B1_yc* gene and the 'native' *CYP1B1_na* gene (co-expressed with only *ΔhRDM* gene), increases over 30 min. Rate was measured in terms of relative fluorescence units (RFUs) using 7-ethoxy resorufin (7-ER) as substrate. 7-ER is de-ethylated by CYP1A1 to form resorufin. (B) Depicts the comparison of fluorescence emitted by the three yeast strains at 30 min of reaction of enzyme with substrate. The data represent mean ± S.D. of three independent experiments.

Indeed, Western blotting (Figure 3.70) provided confirmation that the mRNA derived from *CYP1B1* coding sequence, with yeast biased codons, did translate more protein relative than what was produced by the 'native' mRNA. For this experiment, equal number of cells (1X 10^6), expressing the two variants, 'synthesised' and 'native', of the *CYP1B1* gene were taken for lysis of cells. Densitometric quantification (results not shown) suggests that at least 15 times more protein is produced by *CYP1B1_yc* gene than by the native *CYP1B1_na* gene isolated from a human liver cDNA library.

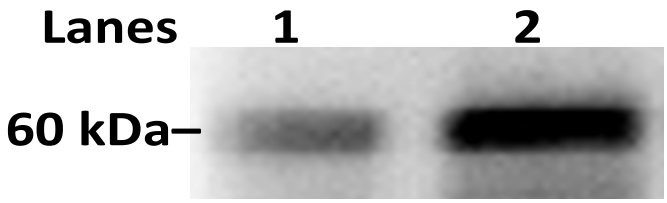


Figure 3.70. Western blot that shows the amounts of CYP1B1 protein being expressed by equal number of cells (1X 10^6) from different yeast strains. Lane 1: protein from cells which contain the plasmid pSYE263/CYP1B1_na (lane 1); lane 2: protein from cells which contain the plasmid pSYE263/CYP1B1_yc (lane 2). The expected size of CYP1B1 protein is 60.8 kDa. The blot was probed by a human CYP1B1 specific monoclonal antibody (Santa Cruz Biotechnology, Cat no: sc-374228).

3.16 Cloning of *CYP4F3A_yc* gene in the episomal plasmid pSY263 for expression of human CYP4F3A enzyme

3.16.1 Cloning of *CYP4F3A_yc* gene in the episomal plasmid pSY263

CYP4F3A is involved in the inactivation leukotriene B4 which is responsible for inflammatory responses within the human body that may lead to cancer and many other diseases (Kalsotra et al., 2004). It catalyses the omega(ω)-hydroxylation of leukotriene B4 to its 20-hydroxy derivative. CYP4F3A belongs to the CYP4 family. Like the other twelve main CYPs, members of this family are also responsible for the bio-activation of various types of medicines that lead to their elimination from the human body. However, they are also involved in the solubilisation of endogenous fatty acids (such as eicosanoids derived from arachidonic acid) which exist within the human body (Kalsotra et al., 2004). Probably because of its difficulty in expression in a recombinant organism, it is not widely available commercially.

The human *CYP4F3A_yc* gene, with yeast biased codons, was cloned in the yeast expression vector pSYE263 using methodologies similar to cloning of the *CYP3A4_yc* gene in the same vector (this Chapter, Section 3.4.1). The resultant plasmid was named pSYE263/h_CYP4F3A_yc.

A 1575 bp *BamH*I-*Xho*I *CYP4F3A_yc* gene fragment (Figure 3.71) was isolated from the plasmid pUC57/BamHI-XhoI/h_CYP4F3A_yc for DNA ligase mediated ligation to the 5699 bp *BamH*I-*Xho*I fragment of vector pSYE263, to create the episomal, 2µ-plasmid pSYE263/h_CYP4F3A_yc (Figure 3.72) for expression of human CYP4F3A enzyme.

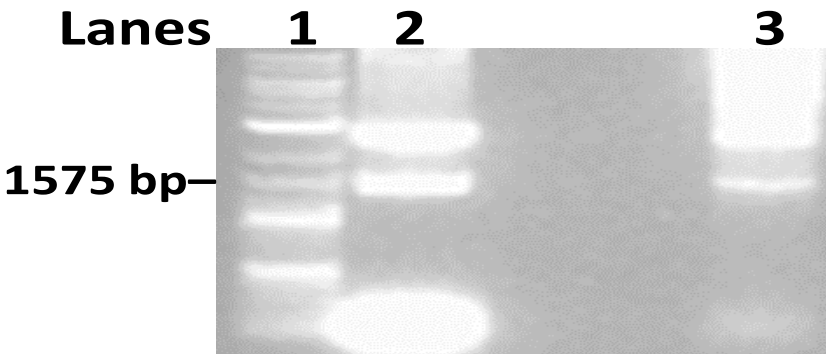


Figure 3.71. An agarose gel that shows the expected DNA fragments when the plasmid pUC57/BamHI-Xhol/h_CYP4F3A_yc was digested with *BamH*I-*Xho*l (lanes 2, 3). The 1575 bp insert fragment was isolated from the gel and ligated with the vector pSYE263 also digested with *BamH*I-*Xho*l to obtain the plasmid pSYE263/h_CYP4F3A_yc.

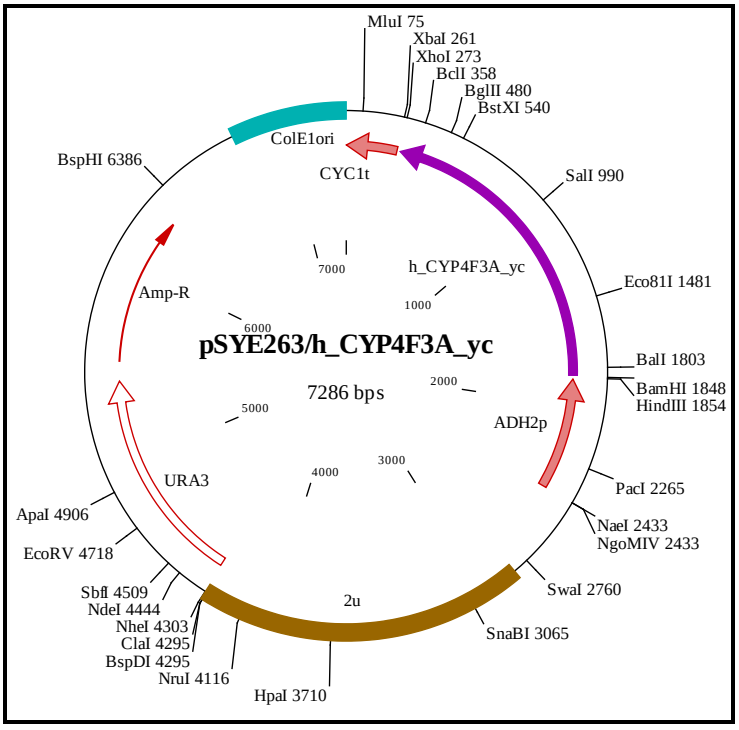


Figure 3.72. The map of the plasmid pSYE263/h_CYP4F3A_yc, with restriction sites that occur in the plasmid only once. The *BamH*I and *Xho*I sites of pSYE263 were used for cloning the synthetic *h_CYP4F3A_yc* gene with yeast biased codons, in the episomal 2µ-plasmid pSYE263.

The authenticity of the resultant plasmid, pSYE263/h_CYP4F3A_yc (Figure 3.72) was confirmed by two separate enzyme digests, with (a) *BamH*I, *Xho*I and (b) *BamH*I-*Xba*I (Figure 3.73).

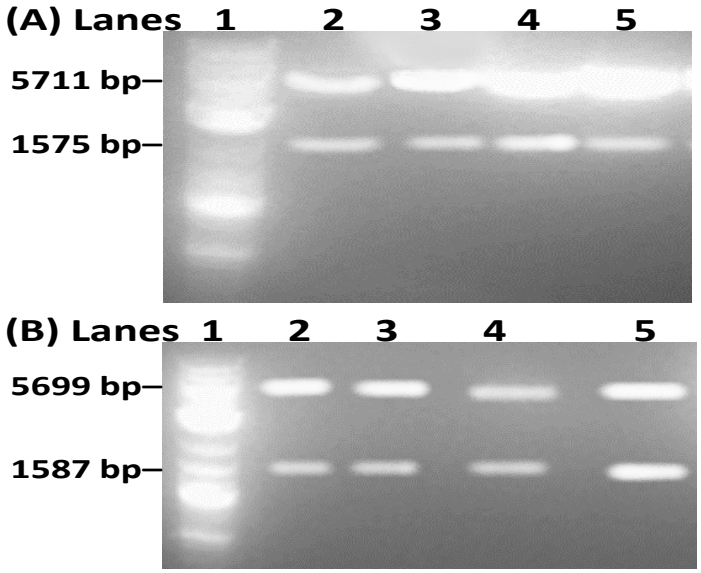
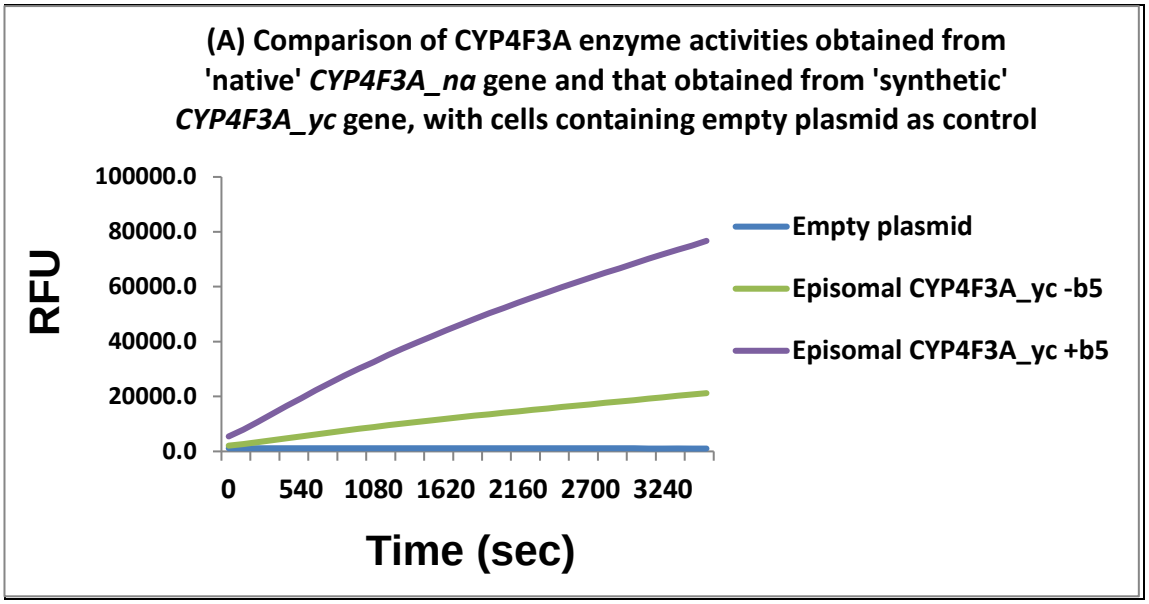


Figure 3.73. Agarose gels that show the expected DNA fragments when the plasmid pSYE263/h_CYP4F3A_yc was digested with restriction enzymes (A) *BamH*I, *Xho*I and (B) *BamH*I, *Xba*I. Lane 1, DNA ladder; lanes 2 to 5, pSYE263/h_CYP4F3A_yc digested with either *BamH*I-*Xho*I (A) or *BamH*I-*Xba*I (B).

3.16.2 Comparison of CYP4F3A enzyme activities expressed by 'synthesised' *CYP4F3A_yc* from an episomal plasmid pSY263, in the presence/absence of cytochrome b5, using a fluorescence-based assay

The results shown in Figure 3.74 (above) indicate that the 'synthesised' *CYP4F3A_yc* gene produces a lot more CYP4F3A active enzyme in the presence of cytochrome b5 (in the strain YAB79) than in its absence (YY7). The 'native' *CYP4F3A_na* gene had not been cloned earlier from a human liver cDNA library. That cytochrome b5 (a) augments CYP4F3A enzyme activity and/ or (b) increases the levels of CYP4F3A protein has not been reported before (Ammed et al., 2016). The possibility of the latter happening can be confirmed by Western blotting.

Indeed, Western blotting (Figure 3.75) has confirmed that cytochrome b5 helps in increasing CYP4F3A protein levels. For this experiment, equal number of cells (1X 10^6), expressing CYP4F3A, in the absence or presence of cytochrome b5, was taken for lysis of cells and extraction of total cellular protein. Densitometric quantification (results not shown) suggested that at least 5 times more protein is produced in the presence of cytochrome b5 than in its absence.



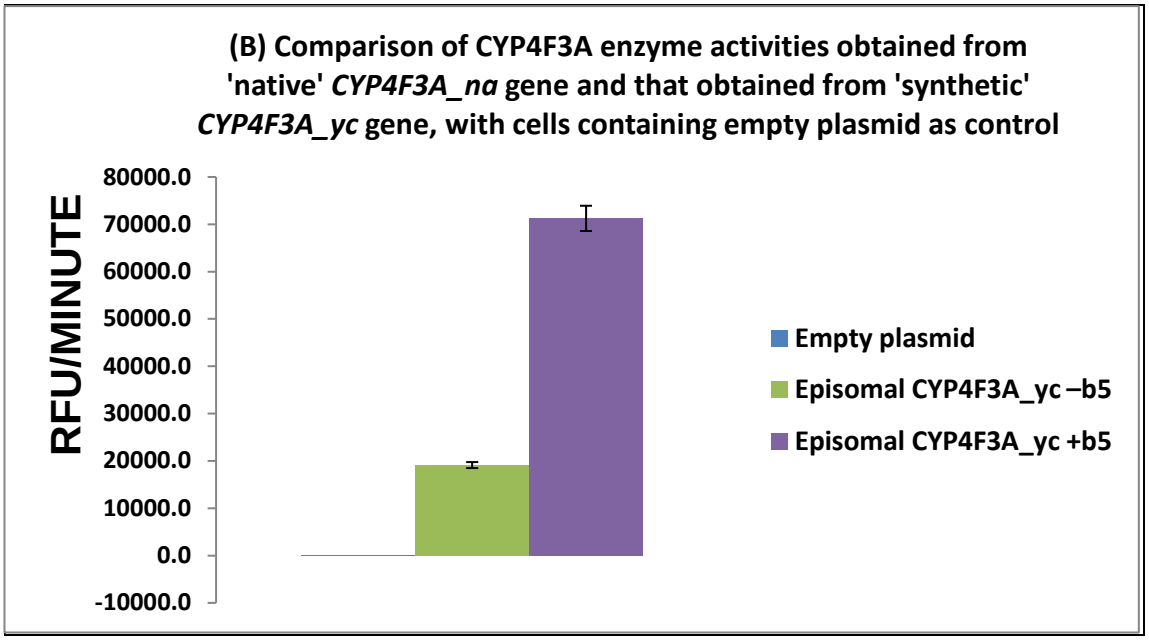


Figure 3.74. (A) The graphs show the rate at which the activity of the CYP4F3A enzyme, expressed in yeast strain YY7 and YAB79 from the 'synthesised' *CYP4F3A_yc* gene [co-expressed only with *ΔhRDM* gene (YY7) or co-expressed with *ΔhRDM* and cytochrome *b5* genes (YAB79)), increases over 40 min. Rate was measured in terms of relative fluorescence units (RFUs) using 3-cyano-7-ethoxycoumarin (CEC) as the substrate. CEC is de-ethylated by CYP4F3A to form 3-cyano7-hdroxycoumarin (CHC). (B) Depicts the comparison of fluorescence emitted by the three yeast strains at 40 min of reaction of enzyme with substrate. The data represent mean ± S.D. of three independent experiments.

Western blot was performed with cells form the same strains used for the experiments described in Figure 3.74. The blot shows that at least 5 times more CYP4F3A protein is produced in the presence of cytochrome b5 than in its absence.

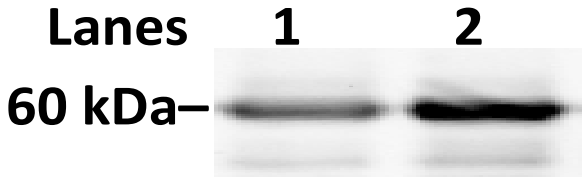


Figure 3.75. Western blot that shows the amounts of CYP4F3A protein which is expressed by equal number of cells (1X 10^6) from two different yeast strains. The amount of total cellular protein obtained from these cells was loaded on wells of an SDS polyacrylamide gel. Cells which contain pSYE263/CYP4F3A_yc + cytochrome b5 (lane 1) and only pSYE263/ CYP4F3A_yc but no cytochrome b5 (lane 2). The expected size of CYP4F3A protein is 59.9 kDa. The blot was probed by a human CYP4F3A specific monoclonal antibody (Santa Cruz Biotechnology, Cat no: sc-374421).

3.17 Cloning of *CYP17A1_yc* gene in the episomal plasmid pSY263 for expression of human CYP17A1 enzyme

3.17.1 Cloning of *CYP17A1_yc* gene in the episomal plasmid pSY263

CYP17A1 plays a key part in the biosynthesis of steroids. Through its ability to control levels of mineralocorticoids, it greatly influences the synthesis of (a) glucocorticoids associated with stress and immune responses and (b) androgens and estrogens which partake in the development and overall homeostasis of reproductive tissues. Hence, it has been reported that because of CYP17A1's multifunctional roles, understanding of its

biochemistry is key to the treatment of prostate cancer, blood pressure, problems related to fertility and other diseases (Mark et al., 2015; Yoshimoto et al., 2015).

The human *CYP17A1_yc* gene, with yeast biased codons, was cloned in the yeast expression vector pSYE263 using methodologies similar to cloning of the *CYP3A4_yc* gene in the same vector (this Chapter, Section 3.4.1). The resultant plasmid was named pSYE263/h_ CYP17A1_yc.

A 1539 bp *BamH*I-*Xho*I *CYP17A1_yc* gene fragment (Figure 3.76) was isolated from the plasmid pUC57/BamHI-XhoI/h_CYP17A1_yc for DNA ligase mediated ligation to the 5699 bp *BamH*I-*Xho*I fragment of vector pSYE263, to create the episomal, 2µ-plasmid pSYE263/h_CYP17A1_yc (Figure 3.77) for expression of human CYP17A1 enzyme.

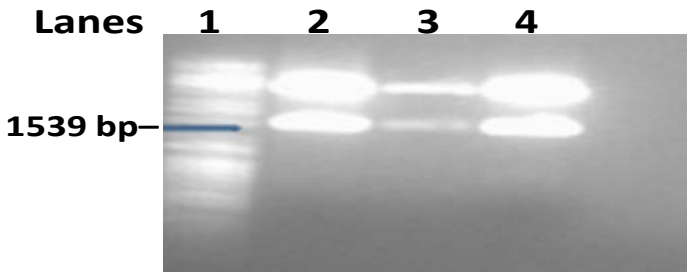


Figure 3.76. An agarose gel that shows the expected DNA fragments when the plasmid pUC57/BamHI-Xhol/h_CYP17A1_yc was digested with *BamH*I, *Xho*I (lanes, 2, 3, 4). The 1539 bp insert fragment was isolated from the gel and ligated to the pSYE263 vector digested with *BamH*I, *Xho*I to obtain the plasmid pSYE263/h_CYP17A1_yc.

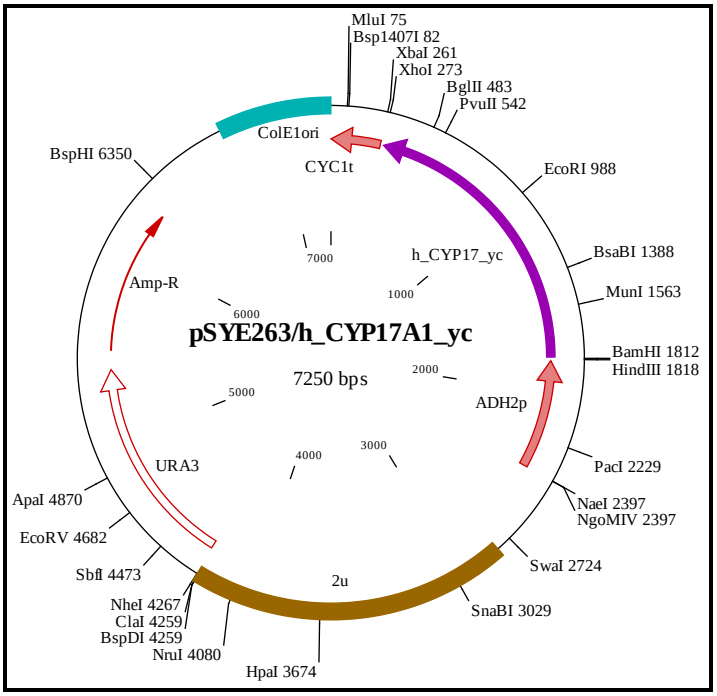


Figure 3.77. The map of the plasmid pSYE263/h_CYP17A1_yc, with restriction sites that occur in the plasmid only once. The *BamH*I and *Xho*I sites of pSYE263 were used for cloning the synthetic *h_CYP17A1_yc* gene with yeast biased codons, in the episomal 2μ-plasmid pSYE263.

After ligation and transformation of the ligation mixture in *E. coli* DH5α competent cells, individual bacterial transformants were grown for preparation of plasmid DNA.

The authenticity of the resultant plasmid DNA, pSYE263/h_CYP17A1_yc was confirmed by digestion with restriction enzymes *BamH*I, *Xho*I. It was verified that the newly constructed plasmid contained the correct size fragments (e.g. 1539 bp *BamH*I, *Xho*I insert; Figure 3.78).

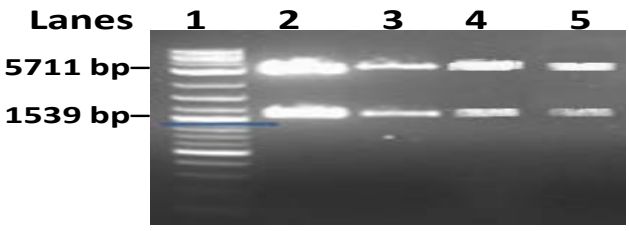


Figure 3.78. An agarose gel that shows the expected DNA fragments when the plasmid pSYE263/BamHI-Xhol/h_CYP17A1_yc was digested with the enzymes BamHI and Xhol (lanes 2 to 5). Lane 1, DNA ladder.

3.17.2 Comparison of CYP17A1 enzyme activities expressed by 'synthesised' *CYP17A1_yc* from an episomal plasmid pSY263, in the presence/absence of cytochrome b5, using a fluorescence-based assay

The results shown in Figure 3.79 (below) indicate that the 'synthesised' *CYP17A1_yc* gene produces a lot more CYP17A1 active enzyme in the presence of cytochrome b5 (in the strain YAB79) than in its absence (YY7). The 'native' *CYP17A1_na* gene had not been cloned earlier from a human liver cDNA library. That cytochrome b5 (a) augments CYP17A1 enzyme activity and/ or (b) increases the levels of CYP17A1 protein has not been reported before (Duggal et al., 2016; Estradal et al., 2103). The possibility of the latter happening can be confirmed by Western blotting.

Indeed, Western blotting (Figure 3.80) has confirmed that cytochrome b5 helps in increasing CYP17A1 protein levels. For this experiment, equal number of cells (1X 10^6), expressing CYP17A1, in the absence or presence of cytochrome b5, was taken for lysis of cells and extraction of total cellular proteins.

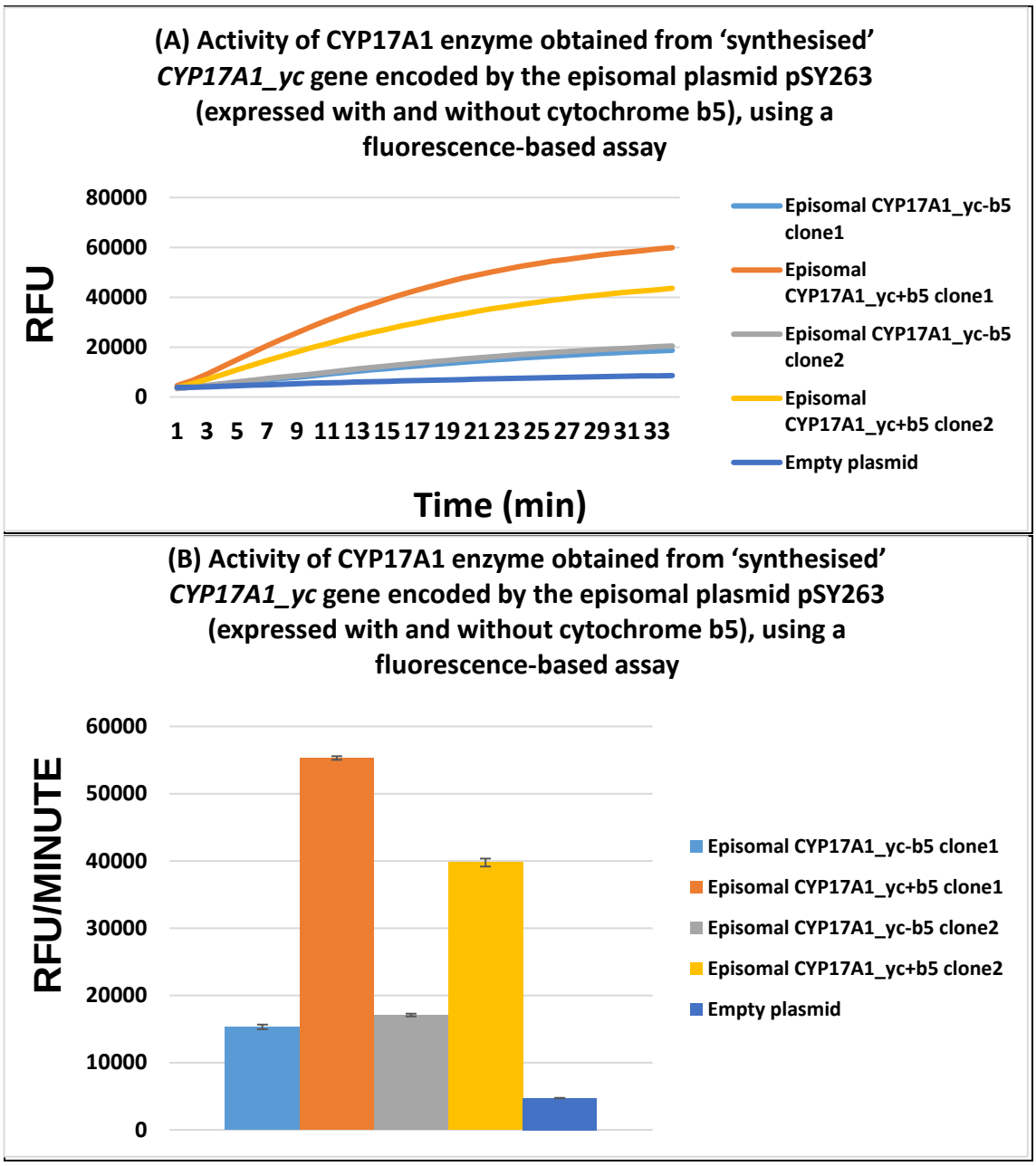


Figure 3.79. The graphs show the rate at which the activity of the CYP17A1 enzyme, expressed in yeast strain YY7 and YAB79 from the 'synthesised' *CYP17A1_yc* gene [co-expressed with only *ΔhRDM* gene (YY7) or co-expressed with *ΔhRDM* and cytochrome *b5* genes (YAB79)), increases over 30 min. Rate was measured in terms of relative fluorescence units (RFUs), using dibenzylfluorescein (DBF) as substrate. DBF is dealkylated by CYP17A1 to form the fluorescent product flourscein. The empty plasmid (pSYE263 containing no CYP17A1 gene), when expressed in yeast, showed no (basal) activity. (B) Depicts the comparison of fluorescence emitted by the different yeast strains at 30 min of reaction of enzyme with substrate. The data represent mean ± S.D. of three independent experiments.

Densitometric quantification (results not shown) of the Western blot (Figure 3.80) suggests that at least 10 times more CYP17A1 protein is produced in the presence of cytochrome b5 than in its absence.

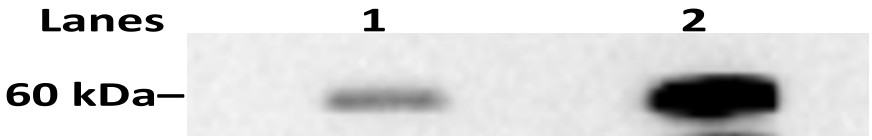


Figure 3.80. Western blot that shows the amounts of CYP17A1 protein being expressed by equal number of cells (1X 10^6) from two different yeast strains. Lane 1: protein from cells which only contain the plasmid pSYE263/CYP17A1_yc but no cytochrome b5 (lane 1); lane 2: protein from cells which contain the plasmid pSYE263/CYP17A1_yc and also cytochrome b5 (lane 2). The expected size of CYP17A1 protein is 57.4 kDa. The blot was probed by a human CYP17A1 specific monoclonal antibody (Santa Cruz Biotechnology, Cat no: sc-374244).

3.18 Cloning of *CYP19A1_yc* gene in the episomal plasmid pSY263 for expression of human CYP19A1 enzyme

3.18.1 Cloning of *CYP19A1_yc* gene in the episomal plasmid pSY263

CYP19A1, also known as aromatase, plays different roles at the crossroads of multiple signalling pathways. It has been claimed that maintenance of its levels is vital for human health. Unfortunately, aromatase concentrations in humans fluctuate dramatically because of environmental chemicals such as chemical additives in food and personal care products which disrupt the endocrine system. Variety of medications and hormone replacement therapy also affect aromatase levels. Aromatase converts androgens to estrogens. It has been reported that alterations of aromatase concentrations can lead to breast, gastric, ovarian, pituitary and prostate cancers, besides many other ailments such as Alzheimer's disease and schizophrenia. It is also thought to be involved in polycystic ovary syndrome, endometriosis and osteoporosis (Petrunak et al., 2014).

The human *CYP19A1_yc* gene, with yeast biased codons, was cloned in the yeast expression vector pSYE263 using methodologies similar to cloning of the *CYP3A4_yc* gene in the same vector (this Chapter, Section 3.4.1). The resultant plasmid was named pSYE263/h_CYP19A1_yc.

A 1524 bp *BamH*I-*Xho*I *CYP19A1_yc* gene fragment (Figure 3.81) was isolated from the plasmid pUC57/BamHI-XhoI/h_CYP19A1_yc for DNA ligase mediated ligation to the 5699 bp *BamH*I-*Xho*I fragment of vector pSYE263, to create the episomal, 2µ-plasmid pSYE263/h_CYP19A1_yc (Figure 3.82) for expression of human CYP19A1 enzyme.

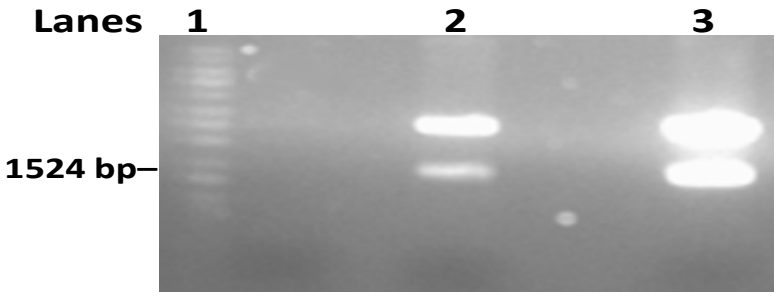


Figure 3.81. An agarose gel that shows the expected DNA fragments when the plasmid pUC57/BamHI-XhoI/h_CYP19A1_yc was digested with *BamH*I, *Xho*I (lanes 2, 3). The 1524 bp gene insert fragment was isolated from the gel and was ligated to the pSYE263 vector that had been digested with *BamH*I, *Xho*I to obtain the plasmid pSYE263/h_CYP19A1_yc.

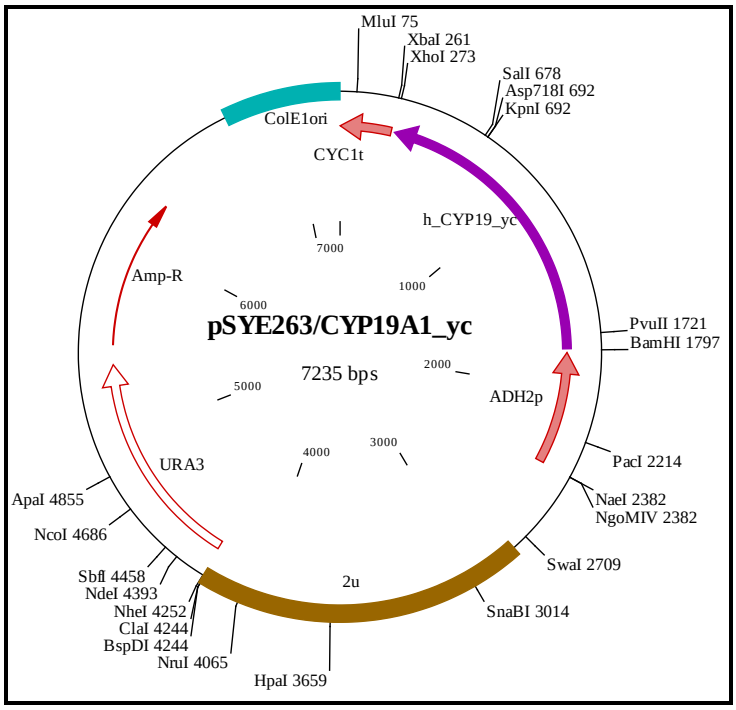


Figure 3.82. The map of the plasmid pSYE263/h_CYP19A1_yc, with restriction sites that occur in the plasmid only once. The *BamH*I and *Xho*I sites of pSYE263 were used for cloning the synthetic *h_CYP19A1_yc* gene with yeast biased codons, in the episomal 2μ-plasmid pSYE263.

After ligation and transformation of the ligation mixture in *E. coli* DH5α competent cells, individual bacterial transformants were grown for preparation of plasmid DNA.

The authenticity of the resultant plasmid DNA, pSYE263/h_CYP19A1_yc was confirmed by digestion with restriction enzymes *BamH*I, *Xho*I. It was verified that the newly constructed plasmid contained the correct size fragments (e.g. 1524 bp *BamH*I, *Xho*I insert; Figure 3.83).

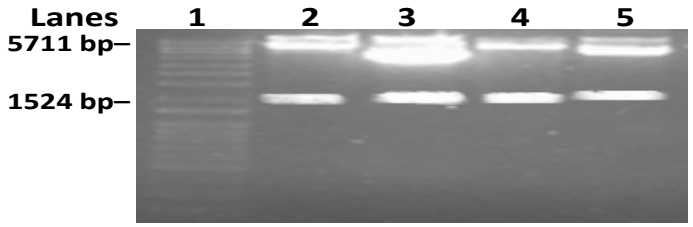


Figure 3.83. An agarose gel that shows the expected DNA fragments when the plasmid pSYE263/BamHI-Xhol/h_CYP19A1_yc was digested with the enzymes BamHI and Xhol (lanes 2 to 5). Lane 1, DNA ladder.

3.18.2 Comparison of CYP19A1 enzyme activities expressed by 'synthesised' *CYP19A1_yc* from an episomal plasmid pSY263, in the presence/absence of cytochrome b5, using a fluorescence-based assay

The results shown in Figure 3.84 (below) indicate that the 'synthesised' *CYP19A1_yc* gene produces as much CYP19A1 active enzyme in the presence of cytochrome b5 (in the strain YAB79) than in its absence (YY7). The 'native' *CYP19A1_na* gene had not been cloned earlier from a human liver cDNA library. In the case of CYP19A1 it would seem that cytochrome b5 may not have any effect on CYP19A1 enzyme activity and/ or levels of CYP19A1 protein. There have been contradictions in the literature regarding the use of cytochrome b5 in the heterologous expression of CYP19A1 (Hiroshi Yamazaki, Tsutomu Shimada, 2006).

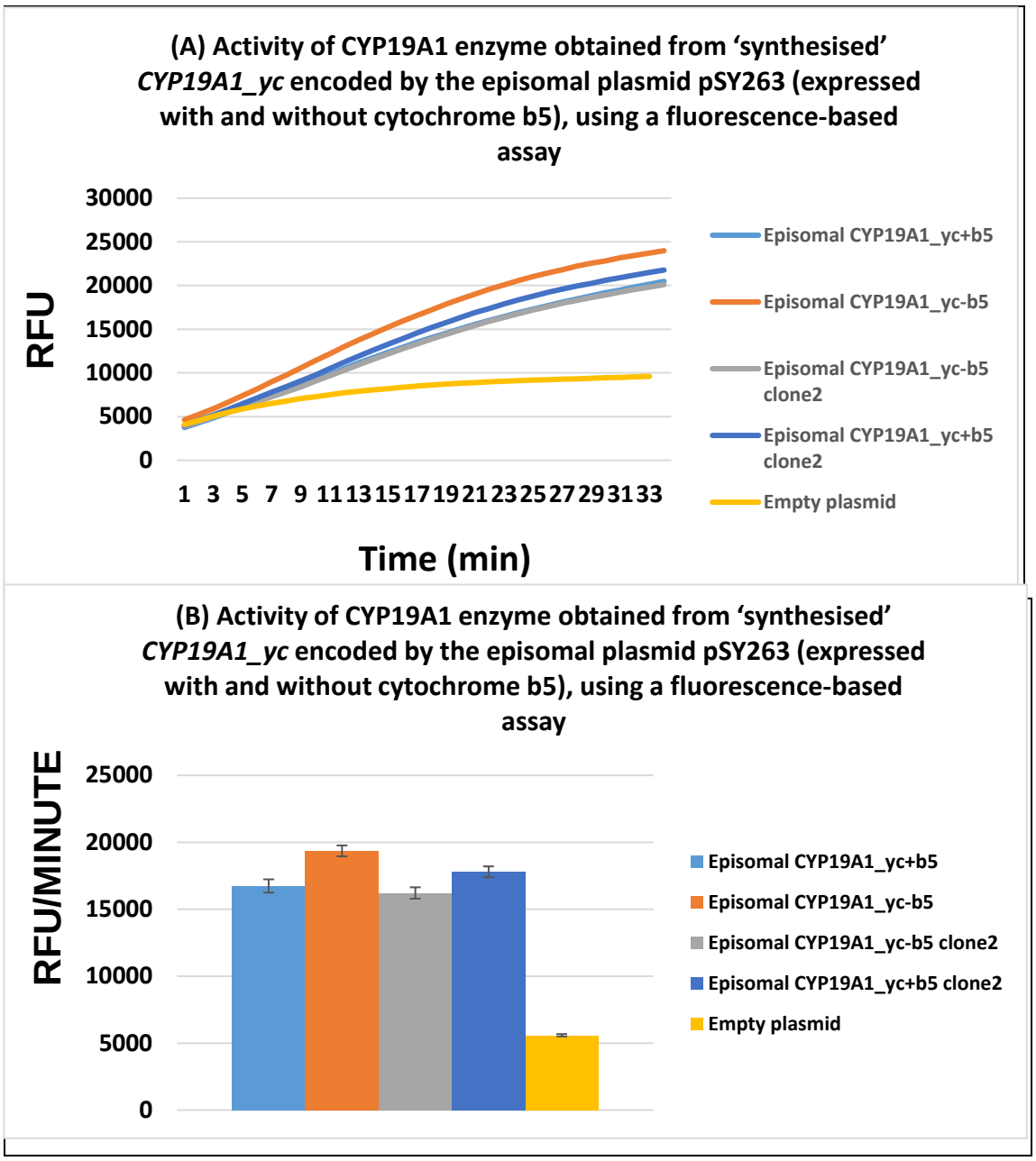


Figure 3.84. The graphs show the rate at which the activity of the CYP19A1 enzyme, expressed in yeast strain YY7 and YAB79 from the 'synthesised' *CYP19A1_yc* gene [co-expressed with only *ΔhRDM* gene (YY7) or co-expressed with *ΔhRDM* and cytochrome *b5* genes (YAB79)), increases over 30 min. Rate was measured in terms of relative fluorescence units (RFUs), using dibenzylfluorescein (DBF) as substrate. DBF is dealkylated by CYP19A1 to form the fluorescent product flourscein. The empty plasmid (pSYE263 containing no CYP19A1 gene), when expressed in yeast, showed no (basal) activity. (B) Depicts the comparison of fluorescence emitted by the different yeast strains at 30 min of reaction of enzyme with substrate. The data represent mean ± S.D. of three independent experiments.

Western blotting (Figure 3.85) confirms that cytochrome b5 may not have much of a role to play in CYP19A1 protein levels during expression in yeast. Densitometric quantification (results not shown) of the Western blot (Figure 3.85) also suggest that is so. For this experiment, once again equal number of cells (1X 10^6), expressing CYP19A1, in the absence or presence of cytochrome b5, was taken for lysis of cells and extraction of total cellular proteins.

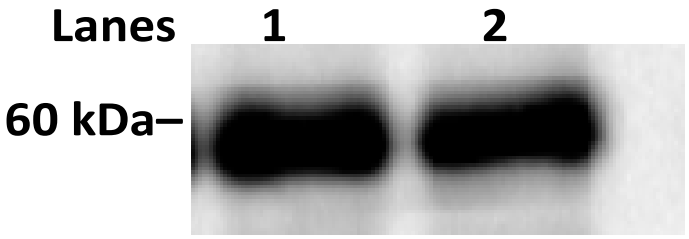


Figure 3.85. Western blot that shows the amounts of CYP19A1 protein being expressed by equal number of cells (1X 10^6) from two different yeast strains. Lane 1: protein from cells which only contain the plasmid pSYE263/CYP19A1_yc but no cytochrome b5 (lane 1); lane 2: protein from cells which contain the plasmid pSYE263/CYP19A1_yc and also cytochrome b5 (lane 2). The expected size of CYP19A1 protein is 52.9 kDa. The blot was probed by a human CYP19A1 specific monoclonal antibody (Santa Cruz Biotechnology, Cat no: sc-374176).

3.19 Cloning of *CYP2J2_yc* gene in the episomal plasmid pSY263 for expression of human CYP2J2 enzyme

3.19.1 Cloning of *CYP2J2_yc* gene in the episomal plasmid pSY263

Like CYP4F3A, CYP17A1 and CYP19A1, CYP2J2 is also an extra-hepatic cytochrome P450 enzyme. It plays a pivotal role in the metabolism of both endogenous (polyunsaturated fatty acids; PUFAs) and exogenous (i.e. xenobiotics) molecules to the human body. In recent years, CYP2J2 has been studied for elucidation of its biological function in cardiac diseases. It has also been noted that CYP2J2 levels are quite high in various cancers. Hence, CYP2J2 modulation has been thought to be a new avenue for treatment of cancer and malfunction of the heart (Ma et al., 2013).

The human *CYP2J2_yc* gene, with yeast biased codons, was cloned in the yeast expression vector pSYE263 using methodologies similar to cloning of the *CYP3A4_yc* gene in the same vector (this Chapter, Section 3.4.1). The resultant plasmid was named pSYE263/h_CYP2J2_yc.

A 1521 bp *BamH*I-*Xho*I *CYP2J2_yc* gene fragment (Figure 3.86) was isolated from the plasmid pUC57/BamHI-XhoI/h_CYP2J2_yc for DNA ligase mediated ligation to the 5699 bp *BamH*I-*Xho*I fragment of vector pSYE263, to create the episomal, 2µ-plasmid pSYE263/h_CYP2J2_yc (Figure 3.87) for expression of human CYP2J2 enzyme.

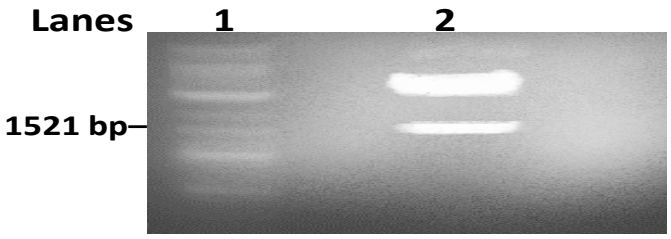


Figure 3.86. An agarose gel that shows the expected DNA fragments when the plasmid pUC57/BamHI-Xhol/h_CYP2J2_yc was digested with *BamH*I, *Xho*I (lanes 2, 3). The 1524 bp gene insert fragment was isolated from the gel and was ligated to the pSYE263 vector that had been digested with *BamH*I, *Xho*I to obtain the plasmid pSYE263/h_CYP2J2_yc.

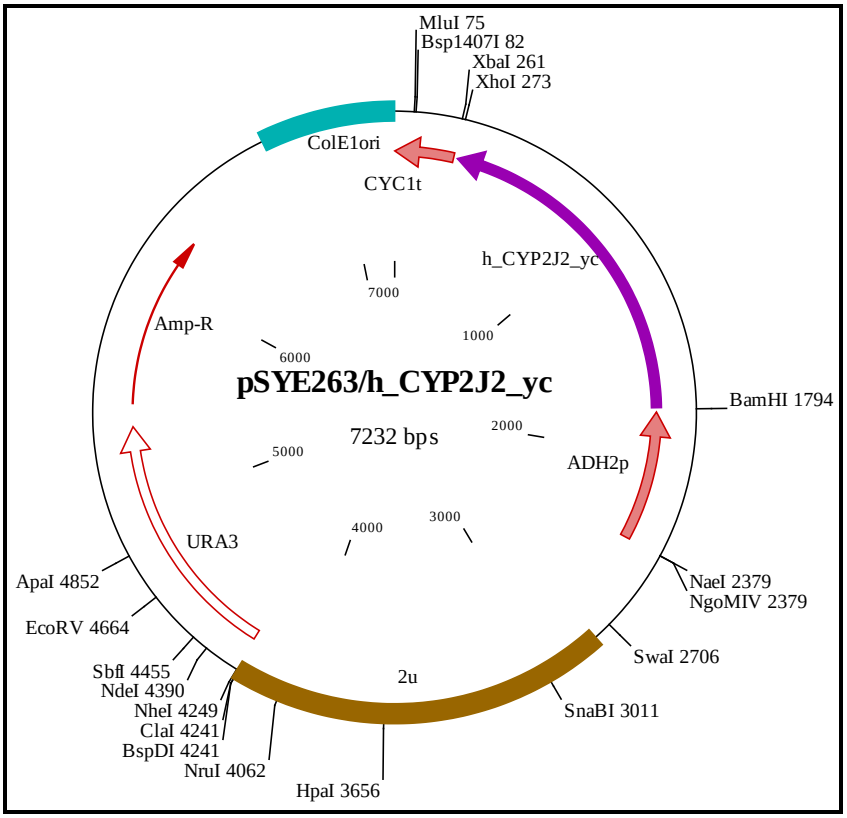


Figure 3.87. The map of the plasmid pSYE263/h_CYP2J2_yc, with restriction sites that occur in the plasmid only once. The *BamH*I and *Xho*I sites of pSYE263 were used for cloning the synthetic *h_CYP2J2_yc* gene with yeast biased codons, in the episomal 2μ-plasmid pSYE263.

After ligation and transformation of the ligation mixture in *E. coli* DH5α competent cells, individual bacterial transformants were grown for preparation of plasmid DNA.

The authenticity of the resultant plasmid DNA, pSYE263/h_CYP2J2_yc was confirmed by two separate enzyme digests, with (a) *BamH*I, *Xho*I and (b) *BamH*I-*Xba*I. It was verified that the newly constructed plasmid contained the correct size fragments Figure 3.88).

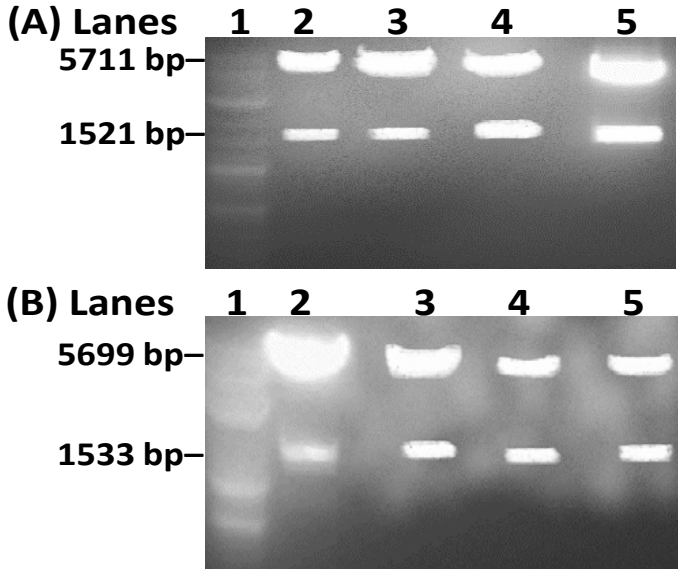


Figure 3.88. Agarose gels that show the expected DNA fragments when the plasmid pSYE263/h_CYP2J2_yc was digested with restriction enzymes (A) *BamH*I, *Xho*I and (B) *BamH*I, *Xba*I. Lane 1, DNA ladder; lanes 2 to 5, pSYE263/h_CYP2J2_yc digested with either *BamH*I-*Xho*I (A) or *BamH*I-*Xba*I (B).

3.19.2 Comparison of CYP2J2 enzyme activities expressed by 'synthesised' *CYP2J2_yc* from an episomal plasmid pSY263, in the presence/absence of cytochrome b5, using a fluorescence-based assay

The results shown in Figure 3.84 (below) indicate that the 'synthesised' *CYP2J2_yc* gene produces as much CYP2J2 active enzyme in the presence of cytochrome b5 (in the strain YAB79) than in its absence (YY7). The 'native' *CYP2J2_na* gene, cloned from a human liver cDNA library, was not available. It seems that cytochrome b5 may not have any noticeable effect on CYP2J2 enzyme activity (Figure 3.89). However, cytochrome b5 does have an influence on the levels of CYP2J2 protein (Figure 3.90). These results would suggest that cytochrome b5 provides stability to the CYP2J2 protein expressed but it may be deleterious to enzyme activity indicating that cytochrome b5 may have an adverse effect on the active site conformation of CY2J2.

Published literature indicates that, until now, only an N-terminal truncated *CYP2J2* gene with C-terminal 6X His-tag has been expressed in bacterial cells but not in yeast (Park et al., 2014). However, it has been noted that there is variability in CYP2J2 enzymatic activity which depends on its redox partners CPR and cytochrome b5. The amounts of CPR and cytochrome b5 co-expressed have dramatic effects on CYPJ2 activity (Evangelista et al., 2013).

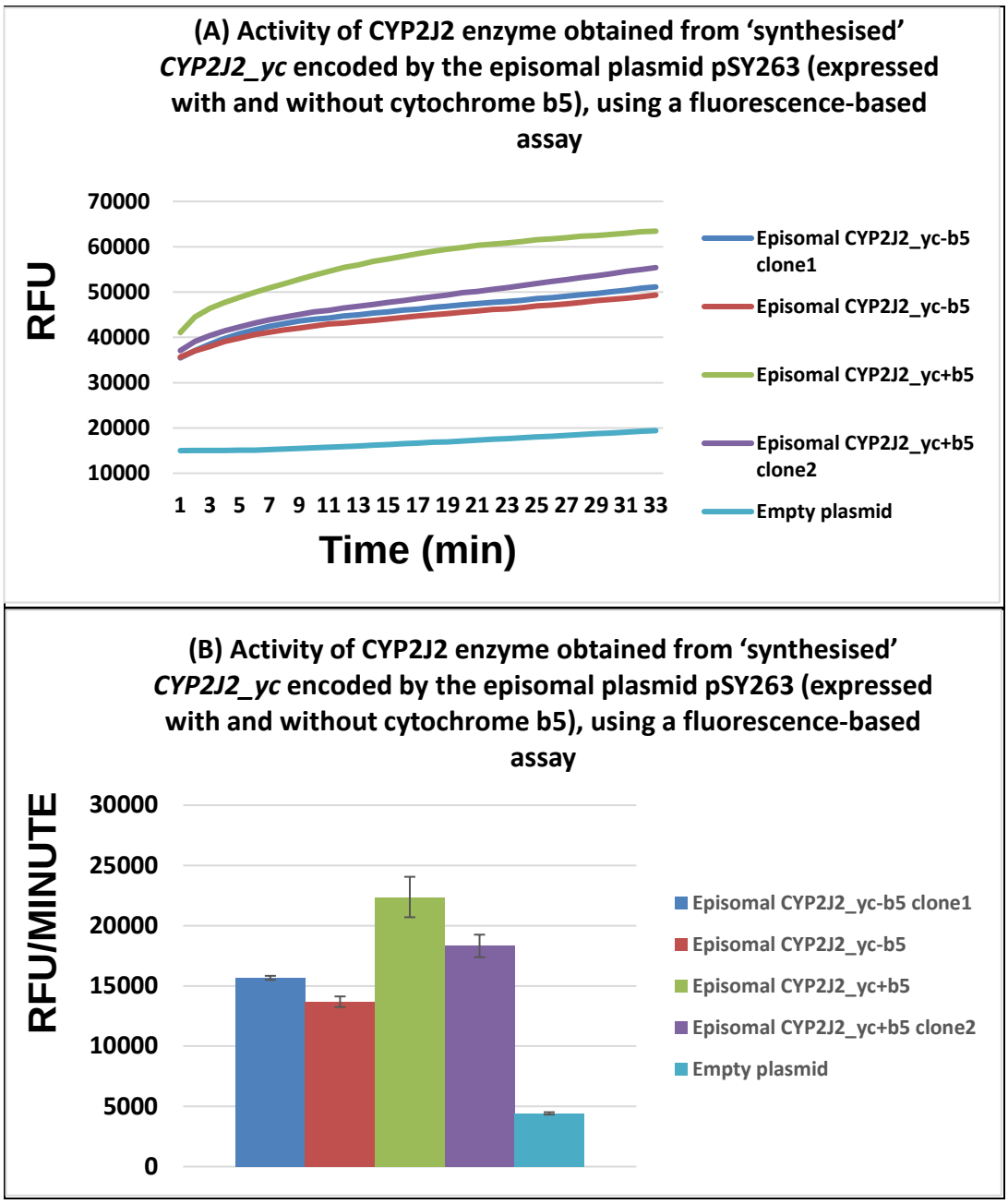


Figure 3.89. The graphs show the rate at which the activity of the CYP2J2 enzyme, expressed in yeast strain YY7 and YAB79 from the 'synthesised' *CYP2J2_yc* gene [co-expressed with only *ΔhRDM* gene (YY7) or co-expressed with *ΔhRDM* and cytochrome *b5* genes (YAB79)), increases over 30 min. Rate was measured in terms of relative fluorescence units (RFUs), using 7-benzyloxymethyloxy-3-cyanocoumarin (BOMCC) as substrate. BOMCC is dealkylated by CYP2J2 to form the fluorescent product 7-hydroxy-3-cyanocoumarin (HCC). The empty plasmid (pSYE263 containing no CYP2J2 gene), when expressed in yeast, showed basal activity. (B) Depicts the comparison of fluorescence emitted by the different yeast strains at 30 min of reaction of enzyme with substrate. The data represent mean ± S.D. of three independent experiments.

Western blotting (Figure 3.90) suggests that cytochrome b5 must have a role to play in CYP2J2 protein levels during expression in yeast. Densitometric quantification (results not shown) of the Western blot (Figure 3.85) suggest that there is 3.5-fold increase in CYP2J2 levels having used total cellular proteins from equal number of cells (1X 10^6) for the blot. Cells which express CYP2J2, in the absence or presence of cytochrome b5, were taken for lysis of cells and extraction of total cellular proteins.

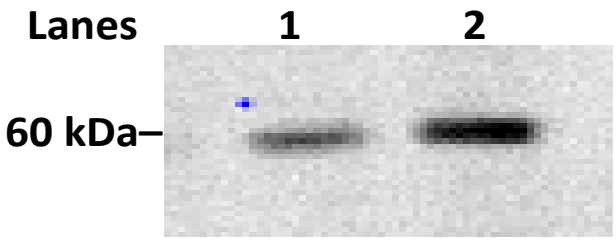


Figure 3.90. Western blot that shows the amounts of CYP2J2 protein being expressed by equal number of cells (1X 10^6) from two different yeast strains. Lane 1: protein from cells which only contain the plasmid pSYE263/CYP2J2 _yc but no cytochrome b5 (lane 1); lane 2: protein from cells which contain the plasmid pSYE263/CYP2J2 _yc and also cytochrome b5 (lane 2). The expected size of CYP2J2 protein is 57.9 kDa. The blot was probed by a human CYP2j2 specific monoclonal antibody (Santa Cruz Biotechnology, Cat no: sc-137127).

3.20 Cloning of *CYP3A5_yc* gene in the episomal plasmid pSY263 for expression of human CYP3A5 enzyme

3.20.1 Cloning of *CYP3A5_yc* gene in the episomal plasmid pSY263

CYP3A5 is another one of the CYP enzymes, which is used for Drug Metabolism studies. It is very similar in its primary sequence to CYP3A4 (Huang et al., 2004; Zanger et al., 2013). At first, the plasmid pUC57/h_CYP35_yc was digested with the restriction enzymes *BamH*I and *Xba*I. The 1521 bp insert fragment containing the human *CYP3A5_yc* gene was isolated from an agarose gel and the 2700 bp pUC57 vector fragment was ignored (Figure 3.91). The *CYP3A5_yc* gene insert was then cloned in pSYE263, which had been digested also with *BamH*I and *Xba*I restriction enzymes to obtain the plasmid pSYE263/h_CYP3A5_yc (Figure 3.92).

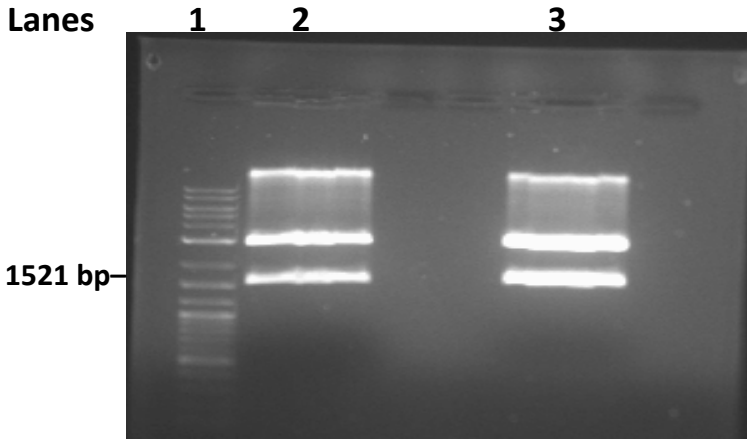


Figure 3.91. An agarose gel that shows the expected DNA fragments when the plasmid pUC57/BamHI-Xhol/h_CYP3A5_yc was digested with *BamH*I, *Xho*I (lanes 2, 3). The 1521 bp gene insert fragment was isolated from the gel and was ligated to the pSYE263 vector that had been digested with *BamH*I, *Xho*I to obtain the plasmid pSYE263/h_CYP3A5_yc.

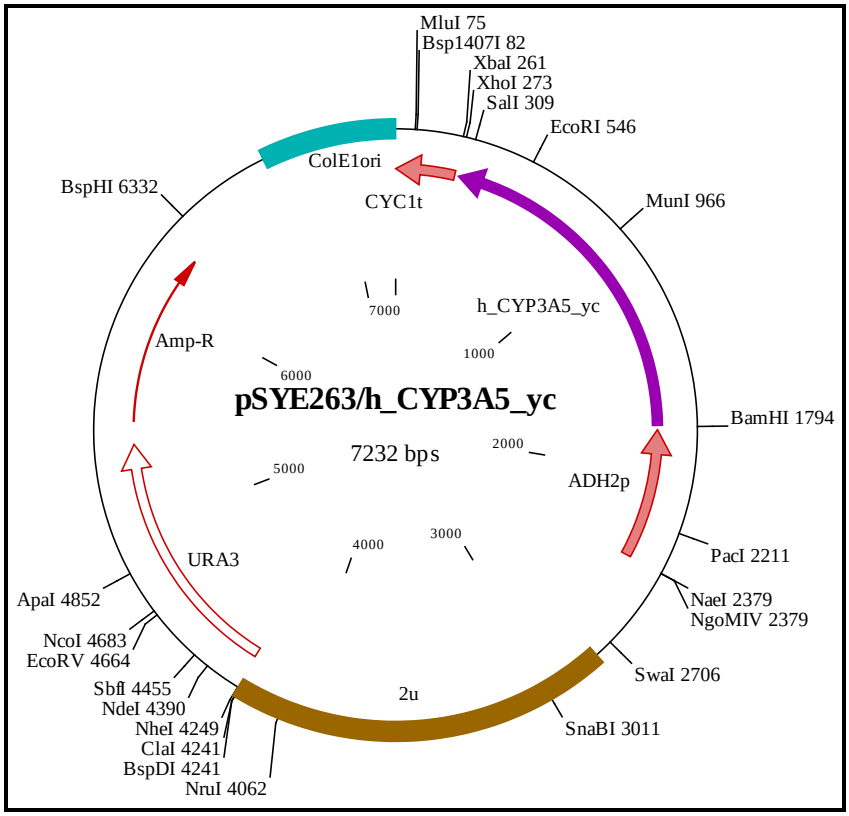


Figure 3.92. The map of the plasmid pSYE263/h_CYP3A5_yc, with restriction sites that occur in the plasmid only once. The *BamH*I and *Xho*I sites of pSYE263 were used for cloning the synthetic *h_CYP3A5_yc* gene with yeast biased codons, in the episomal 2µ-plasmid pSYE263.

After ligation and transformation of the ligation mixture in *E. coli* DH5α competent cells, individual bacterial transformants were grown for preparation of plasmid DNA.

The authenticity of the resultant plasmid DNA, pSYE263/h_CYP3A5_yc was confirmed by digestion with *BamH*I and *Xho*I (Figure 3.93).

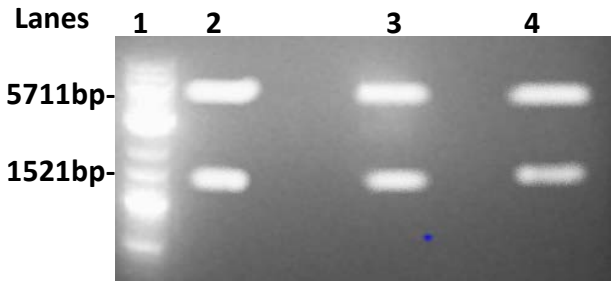


Figure 3.93. An agarose gel that shows the expected DNA fragments when the plasmid pSYE263/h_CYP3A5_yc was digested with restriction enzymes *BamH*I and *Xho*I Lane 1, DNA ladder; lanes 2 to 4, pSYE263/h_CYP3A5_yc digested with *BamH*I-*Xba*I (A).

3.20.2 Comparison of CYP3A5 enzyme activities expressed by 'synthesised' *CYP3A5_yc* from an episomal plasmid pSY263, in the presence/absence of cytochrome b5, using a fluorescence-based assay

The results shown in Figure 3.94 (below) indicate that the 'synthesised' *CYP3A5_yc* gene produces much more CYP3A5 active enzyme in the presence of cytochrome b5 (in the strain YAB79) than in its absence (YY7). The 'native' *CYP3A5_na* gene, cloned from a human liver cDNA library, was not available. It is clear that cytochrome b5 does have a noticeable effect on CYP3A5 enzyme activity indicating cytochrome b5 influences the levels of CYP3A5 protein. Published literature indicates that CYP3A5 makes a substantial contribution to the total metabolic intrinsic clearance of some drugs, and this depends on the presence of at least one wild-type CYP3A5 allele within the cells and the substrate (i.e. the drug) under consideration (Huang et al., 2004).

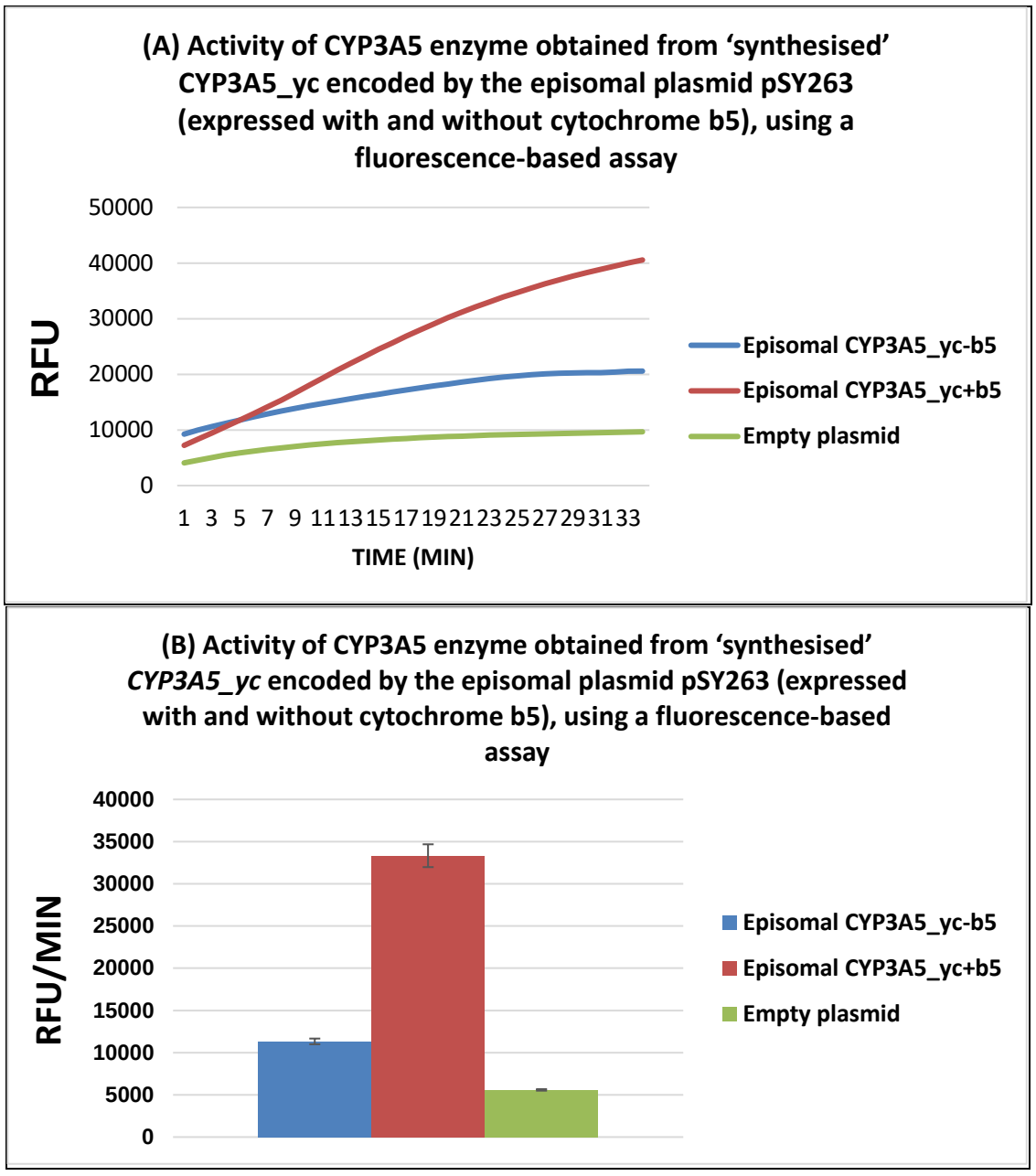


Figure 3.94. The graphs show the rate at which the activity of the CYP3A5 enzyme, expressed in yeast strain YY7 and YAB79 from the 'synthesised' *CYP3A5_yc* gene [co-expressed with only *ΔhRDM* gene (YY7) or co-expressed with *ΔhRDM* and cytochrome *b5* genes (YAB79)), increases over 30 min. Rate was measured in terms of relative fluorescence units (RFUs), using dibenzylfluorescein (DBF) as substrate. DBF is de-alkylated by CYP3A5 to form the fluorescent product flourscein. The empty plasmid (pSYE263 containing no CYP3A5 gene), when expressed in yeast, showed no (i.e. basal) activity. (B) Depicts the comparison of fluorescence emitted by the different yeast strains at 30 min of reaction of enzyme with substrate. The data represent mean ± S.D. of three independent experiments.

Western blotting (Figure 3.95) suggests that cytochrome b5 has a role to play in CYP3A5 protein levels during expression in yeast. Densitometric quantification (results not shown) of the Western blot (Figure 3.95) suggest that there is ~5-fold increase in CYP3A5 levels, in the presence of cytochrome b5, having used proteins from equal number of cells (1X 10^6) for the blot. Cells which express CYP3A5, in the absence or presence of cytochrome b5, were taken for lysis of cells and extraction of total cellular proteins for the Western blot.

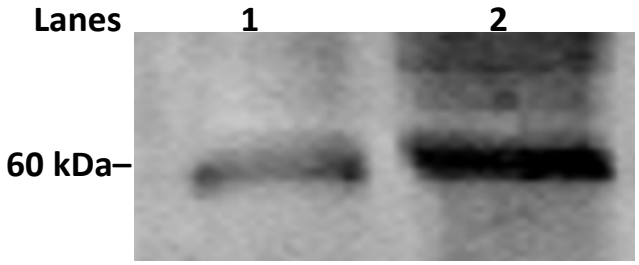


Figure 3.95. Western blot that shows the amounts of CYP3A5 protein being expressed by equal number of cells (1X 10^6) from two different yeast strains. Lane 1: protein from cells which only contain the plasmid pSYE263/CYP3A5 _yc but no cytochrome b5 (lane 1); lane 2: protein from cells which contain the plasmid pSYE263/CYP3A5 _yc and cytochrome b5 (lane 2). The expected size of CYP3A5 protein is 57.1 kDa. The blot was probed by a human CYP3A5 specific monoclonal antibody (Santa Cruz Biotechnology, Cat no: sc-53616).

3.21 Conclusions

Codon usage is a phenomenon defined by 61 codons which code for 20 amino acids (Gray 1999). So far from this Chapter it is clear that, using yeast biased codons, there is up-regulation of the production of CYP450 proteins when they are expressed in baker's yeast. It is not new that fine-tuning of codons for a gene that is expressed in a foreign organism has had a great impact on its expression as a protein. In the past, advances in biotechnology have paved the way for the replacement of rare codons with more frequently occurring ones thereby matching the host organism's codon usage bias (Sharp and Li, 1987). However, the major setback has been the observation that usage of host-specific codons may often have no influence on translation efficiency (Tuller et al., 2010).

In this chapter with the use of yeast biased codons, I have been able to show that:

1. Co-expression of cytochrome b5 has no influence on the activities or levels of expression of CYP2C18 and CYP19A1. Both proteins were co-expressed either (a) with *ΔhRDM* alone or (b) with *ΔhRDM* and cytochrome *b5*. To the best of our knowledge, this has not been published before although observations contrary to ours have been published, i.e. that cytochrome b5 is essential for CYP2C18 activity (Zhang et al., 2015).

2. Co-expression of cytochrome b5 with CYP1A2 enhances levels of protein expression which was confirmed by Western blotting. This has also not been observed before. In fact, it has been mentioned that cytochrome b5 may have a deleterious effect on the expression of CYP1A2 (Zhang et al., 2015).

3. CYPs (2C19, 2C9, 2C8, 3A4, 2A6 and 2B6) have been successfully cloned and

expressed in the baker's yeast strain YAB79 that co-expresses *ΔhRDM* and cytochrome *b5* genes.

4. CYPs (2J2, 17A1 2E1, and 4F3A1) have been cloned and expressed in baker's yeast strain YAB79. All these CYPs have shown that cytochrome b5 is essential for their expression.

5. CYPs (2D6 together with its three SNPs, and 1A1, 1B1) have been successfully cloned and expressed in the baker's yeast strain YY7 that contains only *ΔhRDM*.

In summary, the expression of human *CYP* genes, chemically synthesised using yeast biased codons (i.e. *CYP_yc* genes), have been compared with the expression of CYP cDNAs (*CYP_na* genes) that had been isolated earlier from a human liver cDNA library. It was found that, in general, expression levels of *CYP_yc* genes were much higher than the native *CYP_na* genes. For expression of both sets of genes, an episomal plasmid (usually referred to as a 'multi-copy' plasmid) was used.

The effect of the findings described in this Chapter will be further discussed and utilised in the Chapters that follow. At this stage, one should point out that plasmids derived from an episomal vector are not stable. When expressing genes, encoded by an episomal plasmid, in complete medium which is conducive to high expression, the plasmid is lost. This is because in the absence of selection pressure, an extra-chromosomal plasmid is quickly 'aborted' by the cells. Hence, integrating gene expression cassettes into chromosomal loci could be an alternative that would allow growth of cells and expression of genes in non-selective complete medium. Integration would lead to incorporation of a

gene expression cassette into the genome of yeast and would allow propagation in complete no-selective medium which is far more nutritious than minimal SD medium.

Chapter 4 Improved production of human cytochrome P450 (CYP) enzymes, within yeast cells, from a chromosomally integrated expression cassette

4.1 Introduction

The baker's yeast, *Saccharomyces cerevisiae*, has been the source of wellbeing of humans over a couple centuries from the time it was known that it could be used for baking bread. It also has been used as an immuno-stimulant (Sarlin and Philip 2011).

In the past three decades, baker's yeast, a unicellular eukaryotic microorganism, has widely been exploited by scientists throughout the world for recombinant protein production in both academic labs and commercial projects. Over centuries, this organism has been used for making beer. Elucidation of the genetics behind the beer making process has contributed to the successful use of yeast for the production of recombinant proteins. Kim et al. (2015) recently reported that a protein isolated from baker's yeast shows potential against leukemic cells (Source: Fundacao de Amparo, Sao Paulo, 2017). The human insulin which is sold worldwide by Novo Nordisk is produced from baker's yeast using recombinant DNA technology (Raskin, 1991).

The main characteristic of baker's yeast is its capacity to ferment glucose. It can use the sugar as a sole carbon source for its growth. Ethanol is formed as the only product of fermentation of glucose. For energy conversion, yeast uses two pathways, namely glycolysis and aerobic respiration. The two phases are linked by the use of ethanol as a carbon source in the later phase of growth. In this Chapter, I describe a yeast expression

system that is powered by the promoter of one of the two yeast alcohol dehydrogenase (*ADH*) genes, coding for an enzyme that allows conversion of alcohol into acetaldehyde within yeast cells.

In the 1980s, the two yeast *ADH* genes were identified and were later cloned (Williamson et al., 1980, 1981). The DNA sequences (Russell et al., 1983) revealed the identity of the primary amino acid sequences of the two proteins, Adh1 and Adh2, coded for by the two *ADH* genes. Both proteins consist of 347 amino acid residues. Only 22 residues are different in the two proteins. It has been shown that they have no difference in their ability to use ethanol as a substrate for its biocatalysis to acetaldehyde (Ganzhorn et al., 1987).

In this study, a 573 bp promoter fragment of the yeast *ADH2* gene was isolated from the genomic DNA of the wild type yeast strain S288C (see Chapter 2, Section 2.4.1.3). It was used for construction of plasmids that would allow expression of heterologous human CYP proteins, in yeast. The expression of foreign genes in baker's yeast is usually regulated by constitutive native yeast promoters while inducible promoters have been used to induce high levels of expression of proteins that could potentially be toxic to yeast.

In the past, the inducible copper metallothionein (*CUP1*) and galactosidase (*GAL1* or *GAL10*) promoters (Ramanos et al., 1992) have been utilised for syntheses of a wide range of foreign, mainly human, gene products in baker's yeast. The difficulty with these promoters is that they are not commercially economic. The processes for expression of proteins using these promoters are lengthy, requiring many days. Moreover, the processes are prone to contamination due to the requirement for addition of copper (Cu^{2+}) ions (for *CUP1* promoter) or galactose (for *GAL1*/*GAL10* promoter) which are factors that are

essential for induction of expression of foreign genes which have been cloned downstream of the two inducible promoters.

It has previously been reported that the yeast *ADH2* promoter (ADH2p) is repressed in the presence of glucose and induced in the presence of ethanol (Price et al., 1990). The repression of the mechanism was explained by Di Mauro et al. (2000). In that study, it was explained that as glucose is depleted, the level of the alcohol dehydrogenase regulator (ADR1), a transcription factor, increases. The ADR1 protein then binds to the *ADH2* promoter initiating transcription. Therefore, the most important advantage of the *ADH2* promoter is that no additional inducer is required before or after expression. This has been seen further, during this study, in the case of recombinant yeast cells that were grown over a period of 96 h. During growth of yeast strains that contained one copy, two and three copies of integrated *CYP* gene expression cassettes, only 2% glucose was required every 24 h to supplement growth and for concomitant increase in production of proteins. Over a period of 12 h, glucose is gradually converted into ethanol. During this time period, cells grow vigorously. After complete conversion of glucose to ethanol, the *ADH2* promoter is fully induced by ethanol for production of proteins. During a 96 h time period, CYP activity gradually increases, in parallel with increase in volume of yeast cells (i.e. cell numbers), measured through monitoring of optical density of cells at 600 nm (OD_{600}).

As has been discussed earlier, the presence of a CYP450 reductase (CPR) is essential for CYP enzymatic activity. CPR abstracts electrons from NADPH to the active site of a CYP to allow it to function in the catalysis of a chemical reaction. This implies that, without co-expression of a CPR within host cells, a CYP would be completely inactive. Heterologous expression of certain human CYPs in a host organism, besides requiring a

CPR, also requires co-expression of cytochrome b5 which is the substrate for cytochrome b5 reductase (CBR). Like CPR, CBR also participates in the transfer of electrons to the active site of a CYP (Elahian et al., 2014). It is known from the literature that certain CYPs require the participation of both the CPR and CBR for optimal activity. However, it is not yet clear which specific human CYPs require both CPR and CBR. Cytochrome b5 needs to be co-expressed with CPR and a CYP for CYPs that require CBR. This is because cytochrome b5 is limiting in eukaryotic host organisms (e.g. in insect or yeast cells) that are widely used for recombinant expression of human CYP proteins.

During the studies conducted for this thesis, the role that cytochrome b5 plays in the enzyme activities of the CYP3A proteins, CYP3A4 and CYP3A5, was clarified. It became quite clear that cytochrome b5 is essential for the activity of these two enzymes. It has been reported previously that CYP3A4 catalysed reactions cannot progress without the mediation of cytochrome b5 (Yamazaki et al., 1999, 2002).

This study has also confirmed that co-expression of cytochrome b5 may not be at all necessary, as has been reported earlier, for some of the CYP2C enzymes, i.e. CYP2C19, CYP2C9, CYP2C18 and CYP2C8. The observations reported in this study show for the first time that the CYP2C18 enzyme shows no deficit in its activity, when expressed in the absence of cytochrome b5 (see Chapter 3, section 3.8.2). This is in contrast to what has been published before (Yamazaki et al., 1999, 2002).

This study has also tried to establish which CYP enzyme selectively requires the presence of cytochrome b5. It provides evidence, corroborating earlier claims, that CYP2D6 indeed does not require cytochrome b5 for mediation of its enzyme activity (Yamazaki et al. 2002). However, the activity of CYP1A2 and CYP2E1, quite unexpectedly, is

dramatically enhanced by cytochrome b5 which has never been reported before. Yamzaki et al. (1996) reported in a study that cytochrome b5 stimulation of ferric (Fe^{3+}) CYP450 does not seem to enhance the activity of recombinant human CYPs, CYP1A2 and CYP2E1 (see Chapter 3).

It has been described in Chapter 3 that CYP2D6 enzyme activity decreases 10-12-fold in the presence of cytochrome *b5*. Enzyme assays using recombinant human CYP2D6-bound microsomes, isolated from whole yeast cells, show that the microsomal enzyme may have been damaged by the presence of cytochrome b5 (unpublished results from this lab). In parallel, it was seen that cytochrome b5 greatly enhances CYP1A2 activity within yeast cells and also in recombinant human CYP1A2-bound microsomes that are isolated from yeast cells (see Chapter 5). This was a surprise since it contrasts with the report by Yamazaki et al. (2002) which claims that cytochrome b5 has no stimulatory effect on the activity of CYP1A2. The stimulatory effect of cytochrome b5 in the bio-activation of human CYP1A2 and CYP2E1 has also been studied by Duarte et al (2005) who reported that cytochrome b5 may have a marginal stimulatory effect on CYP1A2 but can profoundly increase the activity of CYP2E1.

One can conclude broadly from the published literature that cytochrome b5 can stimulate reactions mediated by some CYP enzymes and that not all enzymes require the co-expression of this co-factor. However, it is not clear which CYP enzymes require the presence of cytochrome b5. It has been shown in Chapter 3, also for the first time, that cytochrome b5 may be an integral requirement for the 17, 20-lyase activity of the steroidogenic enzyme CYP17A1.

In Chapter 3, the introduction of *CYP* gene bearing episomal 2-micron (2µ)-based plasmids into baker's yeast, through a process known as 'transformation', was described. The results have shown that *CYP* genes, chemically synthesized using yeast-biased codons, produce higher enzyme activities compared to the enzymes produced from native genes isolated from a liver cDNA library. An episomal plasmid can be maintained in selective minimal media which lack the nutrition for vigorous growth of yeast cells. Production of a heterologous protein in yeast using a minimal selective medium often becomes a futile exercise since very little protein, in our case, CYP enzyme is obtained because of poor cell growth. Instead, complete full medium which does not select for the presence of the plasmid bearing the heterologous (in our case *CYP*) gene, is used for short term expression over a period of 24 to 48 h. However, in full medium, there is always huge loss of plasmid from the cell population in a few generations of growth, implying that cells continue to grow in the absence of a plasmid that bears the heterologous (*CYP*) gene.

To avoid plasmid loss and allow growth of yeast cells in complete full medium, we have established a generic technology that allows chromosomal integration of CYP expression cassettes bearing *CYP* genes, synthesized using yeast bias codons, to create highly stable yeast strains that can continuously express *CYP* genes of interest over many generations merely by continuous feeding the cells with the nutrients in the full media. Since the promoter used for gene transcription is *ADH2*, the duration of growth of yeast cells could be as long as the cells can divide within a defined enclosed space (i.e. a fermentor). It should be noted that aged cells are incapable of vigorous cell division. For stoppage of cell growth, because of aging, could take more than a fortnight, perhaps much longer, depending on the conditions used for fermentation. Moreover, in order to prevent cells to

become fully saturated with ethanol whereby they cannot grow any further, cells can be grown under fed-batch fermentation conditions, where ethanol produced is drained away and glucose is continuously drip-fed.

In this Chapter, *ADH2* promoter driven expression of CYPs has been monitored within live cells using substrates which make fluorescent products upon reaction with active CYP enzymes. Based on CYP activities, levels of expression of CYPs have thus been compared rapidly.

4.2 A typical cellular assay that allows monitoring of CYP activities to permit comparison of CYP expression levels between different recombinant yeast strains

Yeast strains harbouring *CYP* genes, stored at -80°C, freezer were streaked out on minimum media agar plates containing different selection markers. The plates were grown for 3 days, at 30°C, in a static incubator. A scoopful of cells which grew on plates was taken for inoculation in full YDP broth, which is a complex rich medium for routine yeast growth. It contains 1% bacto-yeast extract, 2% bacto-peptone, 2% glucose, as described in Chapter 2. It cannot select for the presence of extra-chromosomal plasmids within cells.

5 ml of YPD broth was transferred into 6-well sterile plastic plates. Then, the inoculum (i.e. scoopful of cells) from freshly grown agar plates, containing different yeast strains, was added to the different wells of a 6-well plate containing 1 ml of YPD broth. 2%

adenine was added was added to supplement the adenine present in YPD because adenine is rapidly used-up by the cells' biomass.

The 6-well plates were transferred to a shaking incubator set at 30°C for overnight growth of cells. After 12 h, glucose is used up and full induction of the *ADH2* promoter occurs. This allows expression of the CYP enzyme. The optical density at 600 nm (i.e. OD_{600}) was measured from each of the incubated cultures. Cells usually attained an OD_{600}/ml of around 25. The cells were transferred into 2 ml Eppendorf tubes and were harvested. The cell pellets were washed three times with 500 µl TE buffer (50 mM Tris-HCl, 1 mM EDTA, pH 7.4) by spinning down the cells at 13,000 rpm, each time for 1 min. Cells were finally re-suspended in 450 µl of TE buffer to measure the kinetics of CYP enzyme activities.

4.3 Assay for CYP enzymes activities

50 µl of cell suspensions were transferred into a sterile 96-well microtiter plate to which 50 µl of a substrate mixture (depending on the substrate for a specific CYP) was added. The parameters for the fluorescence plate reader (Synergy HT BioTek) were set using the appropriate extinction/emission filters and appropriate gain sensitivity setting to obtain the best kinetic output from the plate reader. The plate was incubated at 30°C for 30 sec before the fluorescence emissions were measured using specific fluorogenic substrates (Table 4.1).

Table 4.1. Fluorogenic substrates used for monitoring cellular CYP enzyme activities.

CYPs	Non-Fluorescent Substrates	Fluorescent Product Formed
3A4, 2C9, 3A5, 2C8	DBF	Fluoroscein
2D6,2E1	EOMCC	7-HCC
4F3A	CEC	CHC
1A2, 2C19,	CEC	CHC
1A1, 1B1	EROD	Resorufin
2C9	7-MFC	HFC

4.4 Outline of Chapter 4

This chapter describes the expression of a copy of synthetic *CYP* gene (chemically synthesized with yeast-biased codons), from a single yeast 'chromosomal' locus. The aim was to find out which chromosomal locus gives the best expression of human CYP proteins. Expression of genes from chromosomal loci allows growth of yeast cells in

cheap, ‘non-selective’ growth media, continuously over 5 to 7 days, or longer, in shake-flasks. As described above, it could be far longer in appropriate fermentors. In contrast, expression from extra-chromosomal, episomal plasmids demand growth of yeast cells in ‘selective’ growth media, which are expensive, where cell numbers are relatively low, and cell growth is restricted to 24 – 48 h.

It has been speculated that, heterologous (i.e. foreign) gene expression from yeast depends on the yeast proteins that reside in the neighbourhood of the human protein that is being expressed from a particular yeast chromosome (Branco and Pombo, 2006; Romanos et al., 1992). Hence, CYP gene expression cassettes (consisting of a promoter, a gene of interest and a transcription terminator) were integrated into different chromosomal loci, using homologous recombination, a technology which also facilitates gene therapy in human cells. The results obtained clearly show that there is differential expression of a human CYP enzyme when expressed from the neighbourhood of the yeast *ADE2*, *HIS3* and *URA3* gene locations. Indeed, the best human CYP expression occurs from a particular locus of chromosome XV, where the yeast *HIS3* gene resides.

Integrative plasmids that encode the *CYP_yc* genes were constructed so that a copy of a *CYP_yc* gene could be integrated into 2 or 3 different yeast chromosomal loci. Expression of a CYP enzyme activity from a yeast strain that contained an integrated copy of a *CYP_yc* gene were then compared with the strain that harboured an episomal plasmid encoding the same *CYP_yc* gene.

4.5 Construction of yeast integrative plasmids that would allow integration of CYP gene expression cassettes into the yeast genome

4.5.1 Construction of the yeast integrative plasmid YIpAdeADH2S to allow integration at the ADE2 locus of the yeast genome

The yeast integrative plasmid YIpAdeADH2S (Y= yeast; I= integrative; p= plasmid; Ade= *ADE2* selection marker; ADH2= *ADH2* promoter; S= *SUC2* terminator sequence) was created via the following steps (1) to (3).

(1) Isolation of vector fragment.

The plasmid YIpAdeGAL1S (Figure 4.1) contains the *GAL1* promoter (GAL1), which had been isolated from the *GAL1*/*GAL10* gene of baker's yeast, *S. cerevisiae*, by PCR using genomic DNA as a template. The promoter can drive expression of genes which are cloned downstream at its 3'-end. 'S' signifies the *SUC2* terminator, a transcription termination signal isolated from the *SUC2* gene of *S. cerevisiae* by PCR, using genomic DNA as a template. The plasmid YIpAdeGAL1S was digested with the restriction enzymes *BamH*I and *Sal*I to obtain the 5798 bp 'YIpAde' fragment (Figure 4.2). It was isolated and used as the vector for ligation, as in step (3). Restriction enzyme digestion with *BamH*I, *Sal*I eliminates the *GAL1* promoter and allows, in its place, introduction of the *ADH2* promoter.

(2) Isolation of insert fragment.

The plasmid pBluKS(+)/ADH2p (Figure 4.3) was digested with *BamH*I, *Sal*I. The 573 bp *ADH2* promoter (ADH2p) insert fragment was isolated (Figure 4.2). The ADH2p fragment had originally been isolated from the genome of *S. cerevisiae* by PCR.

(3) The vector and insert fragments, from (1) and (2), were ligated to obtain the plasmid YIpAdeADH2S (Figure 4.4).

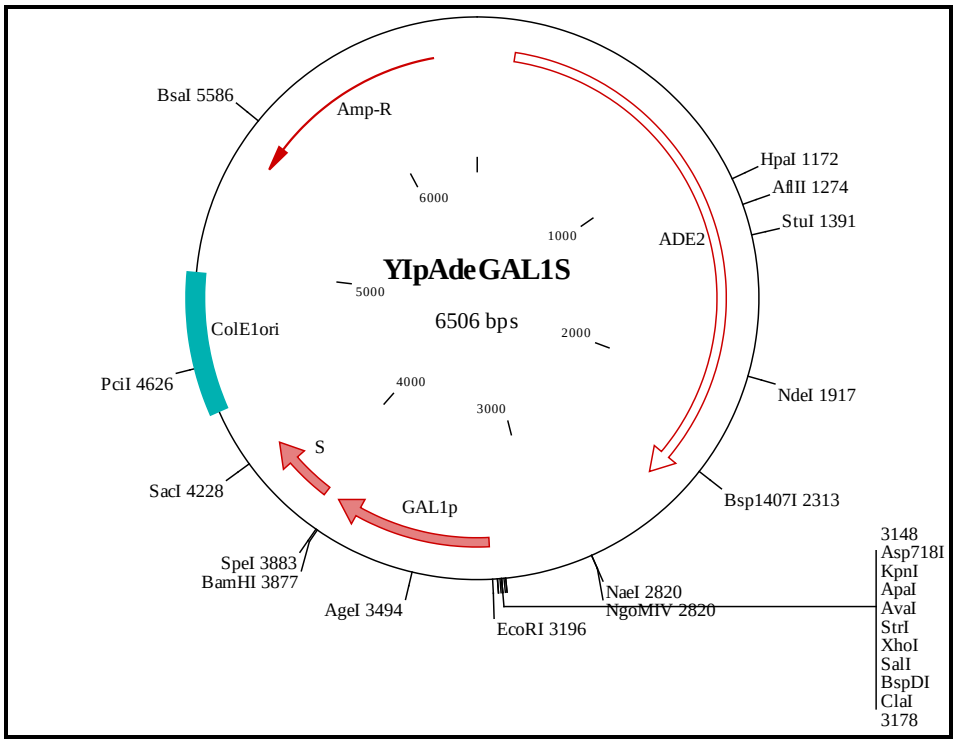


Figure 4.1. Map of YIpAdeGAL1S that contains the GAL1 promoter and which allows integration pf the plasmid and its derivatives into the ADE2 locus of the yeast genome. The restriction sites shown are the ones that occur only once in the plasmid.

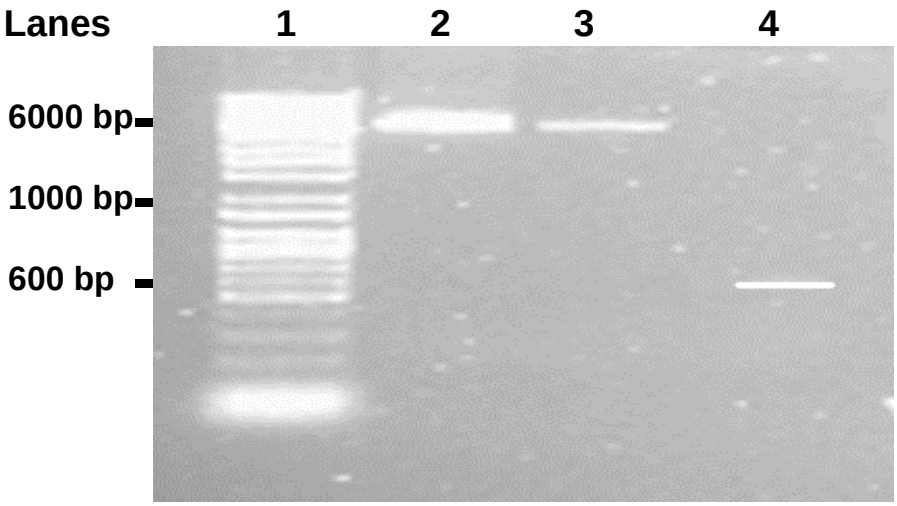


Figure. 4.2. The agarose gel shows the 5798 bp vector fragment obtained after digestion of the plasmid YIAdeGAL1S with the restriction enzymes BamHI and SalI (lanes 2, 3). Lane 4, The 593 bp ADH2p insert fragment obtained after digestion of plasmid pBluKS(+)/ADH2p (Figure 4.3) with BamHI, SalI. The fragments were isolated from the agarose gel for further ligation. Lane 1, 2-log DNA ladder.

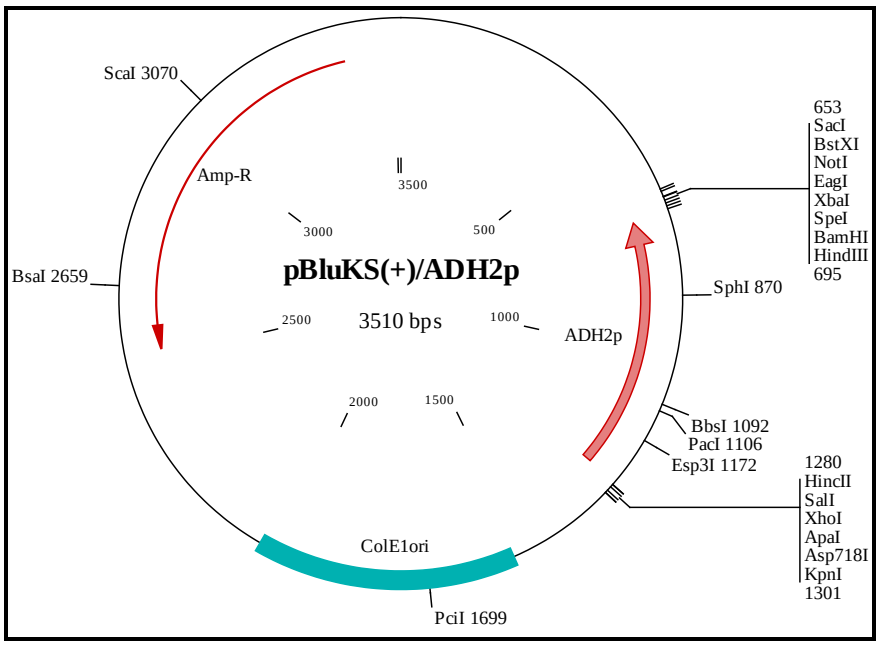


Figure 4.3. The map of the plasmid pBluKS(+)/ADH2p from which the ADH2 promoter fragment was isolated. It shows restriction sites that occur in the plasmid only once.

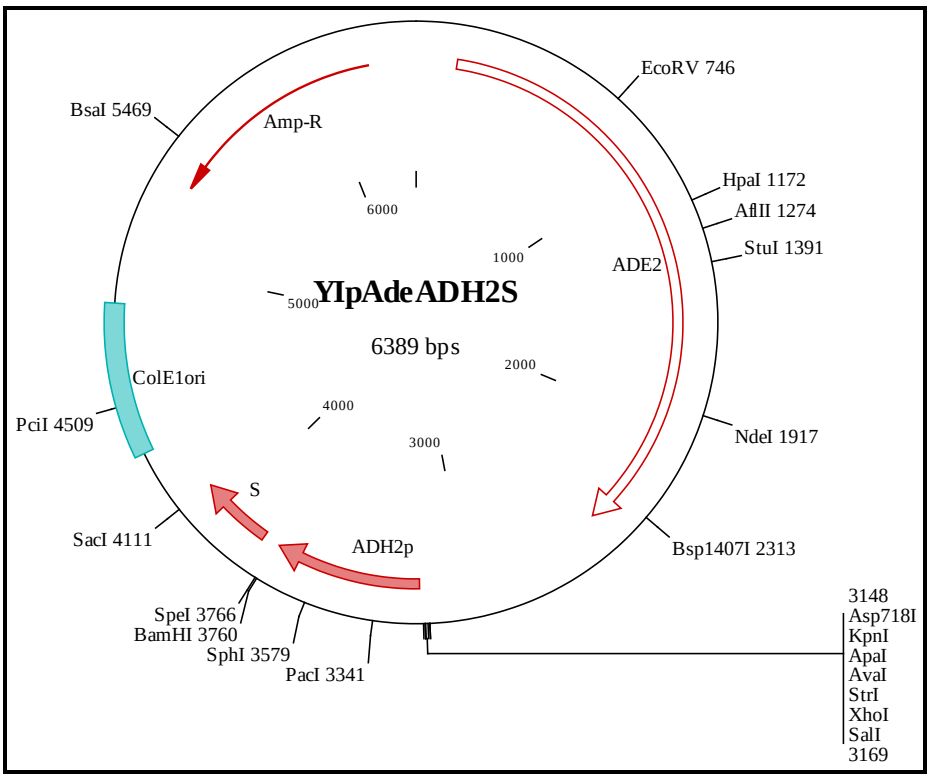


Figure 4.4. The map of the plasmid YIpAdeADH2S. It contains the ADH2 promoter (ADH2p) and the SUC2 terminator (S). The plasmid and its derivatives would allow integration into the ADE2 locus of the yeast genome. The map shows restriction sites that would cut the plasmid only once.

The 5798 bp YIAde vector fragment and the 593 bp ADH2p insert were ligated with the help of the enzyme, DNA ligase. After ligation and transformation in the *E. coli* strain DH5α, 3 individual colonies were grown for preparation of plasmid DNA using the alkaline lysis method (Chapter 2; Section2.4.1.8). The resultant plasmid, YIAdeADH2MS (Figure 4.4), was confirmed by multiple restriction enzyme digestions. The agarose gel that shows digestion of the plasmid with restrictions enzymes, *BamH*I, *Sal*I are shown in Figure 4.5. The expected fragments were observed confirming the integrity of the newly created plasmid.

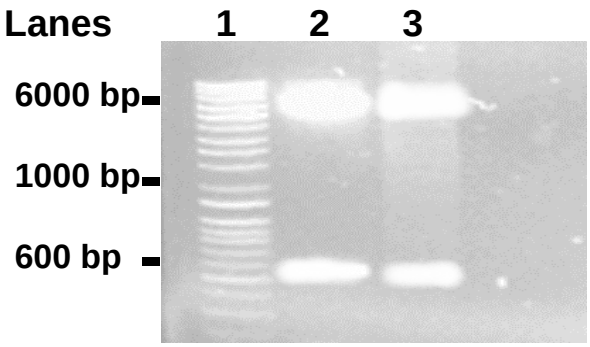


Figure 4.5. An agarose gel that shows the expected fragments (5798 bp and 593 bp) of different clones of the plasmid YIpAdeADH2S, after digestion with restriction enzymes BamHI and SalI (lanes 2, 3). Lane 1, 2-log DNA ladder.

The plasmid YIpADE2ADH2S can now be used for cloning of any gene downstream of the *ADH2* promoter. The gene expression cassettes in the resultant plasmids can then be integrated into the *ADE2* locus on chromosome XV (564476to 566191bps) of the yeast *S. cerevisiae*.

4.5.2 Construction of the yeast integrative plasmid, YIpTrpADH2S, that allows integration of a CYP gene expression cassette at the TRP1 locus of the yeast genome

The yeast integrative plasmid YIpTrpADH2S (the letters in the name of the plasmid has the same meaning as that of the plasmid described in Section 4.4.1, excepting that 'Trp' stands for the functional *TRP1* gene, was created via the following steps:

(1) Isolation of vector fragment.

The plasmid YIpTrpGAL1S (Figure 4.6) contains the *GAL1* promoter (GAL1). The plasmid was digested with *BamH*I and *Sal*I to obtain the 4544 bp 'YIpTrp' fragment which was isolated and used for ligation, as in step (3). Restriction

enzyme digestion with *BamH*I, *Sal*I eliminated the *GAL1* promoter to allow its replacement by the *ADH2* promoter.

(2) Isolation of insert fragment.

The 593 bp *ADH2* promoter (ADH2p) insert fragment was isolated from the plasmid pBluKS(+)/ADH2p (Figure 4.3), as in Section 4.4.1.

(3) The vector and insert fragments, from (1) and (2), were ligated to obtain the plasmid YIpTrpADH2S (Figure 4.7).

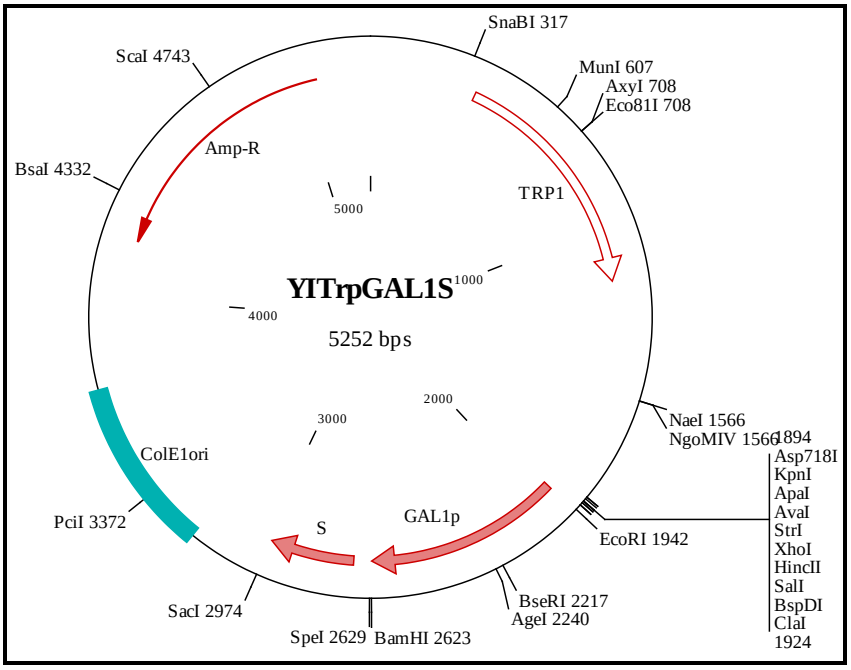


Figure 4.6. Map of YIpTrpGAL1S that contains the *GAL1* promoter and which allows integration of the plasmid and its derivatives into the TRP1 locus of the yeast genome. The restriction sites shown are the ones that occur only once in the plasmid.

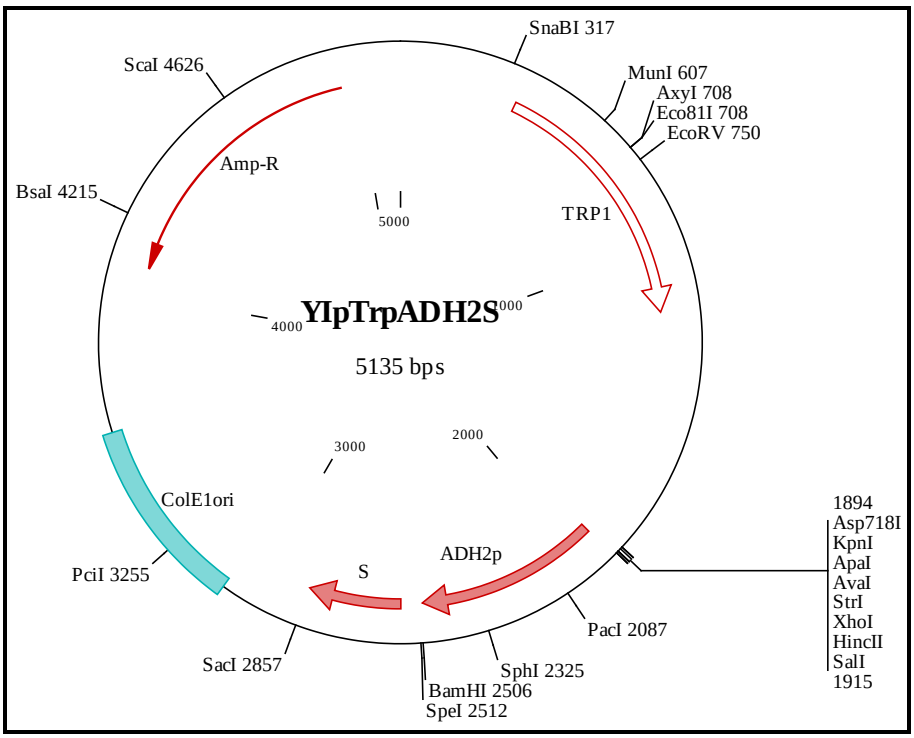


Figure 4.7. The map of the plasmid YIpTrpADH2S. It contains the *ADH2* promoter (ADH2p) and the SUC2 terminator (S). The plasmid and its derivatives would allow integration into the *TRP1* locus of the yeast genome. The map shows restriction sites that would cut the plasmid only once.

The resultant plasmid, YITrpADH2MS (Figure 4.7), was confirmed by two sets of restriction enzyme digests (Figure 4.8).

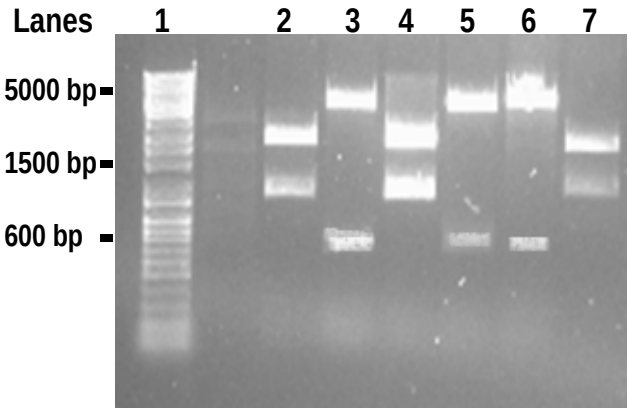


Figure 4.8. An agarose gel that shows the expected fragments of different clones of the plasmid YIpTrpADH2S after digestion with (a) restriction enzymes BamHI and SalI (4544, 593 bp; lanes 3, 5, 6) and (b) restriction enzyme PvuII (3826, 1309 bp; lanes 2, 4, 7). Lane 1, 2-log DNA ladder.

The results obtained from the agarose gel (Figure 4.8) confirm the integrity of the newly created plasmid.

The YIpTrpADH2S plasmid can now be used for cloning any gene downstream of the *ADH2* promoter. The gene expression cassettes in the resultant plasmids can then be integrated into the *TRP1* locus on chromosome IV (461842 to 462516bps) of the yeast *S. cerevisiae*.

4.5.3 Construction of the yeast integrative plasmid, YIpHisADH2S, that allows integration of a CYP gene expression cassette at the HIS3 locus of the yeast genome

The yeast integrative plasmid YIpHisADH2S (the letters in the name of the plasmid has the same meaning as that of the plasmid described in Section 4.4.1, excepting that 'His' stands for the functional *HIS3* gene) was created via the following steps:

(1) Isolation of vector fragment.

The plasmid YIpHisGAL1S (Figure 4.9) contains the *GAL1* promoter (GAL1). The plasmid was digested with *BamH*I and *Sal*I to obtain the 4726 bp 'YIpHis' fragment (Figure 4.10) which was isolated and used for ligation, as in step (3). Restriction enzyme digestion with *BamH*I, *Sal*I eliminated the *GAL1* promoter to allow its replacement by the *ADH2* promoter.

(2) Isolation of insert fragment.

The 593 bp *ADH2* promoter (ADH2p) insert fragment was isolated from the plasmid pBluKS(+)/ADH2p (Figure 4.3), as in Section 4.4.1.

(3) The vector and insert fragments, from (1) and (2), were ligated to obtain the plasmid YIpHisADH2S (Figure 4.11).

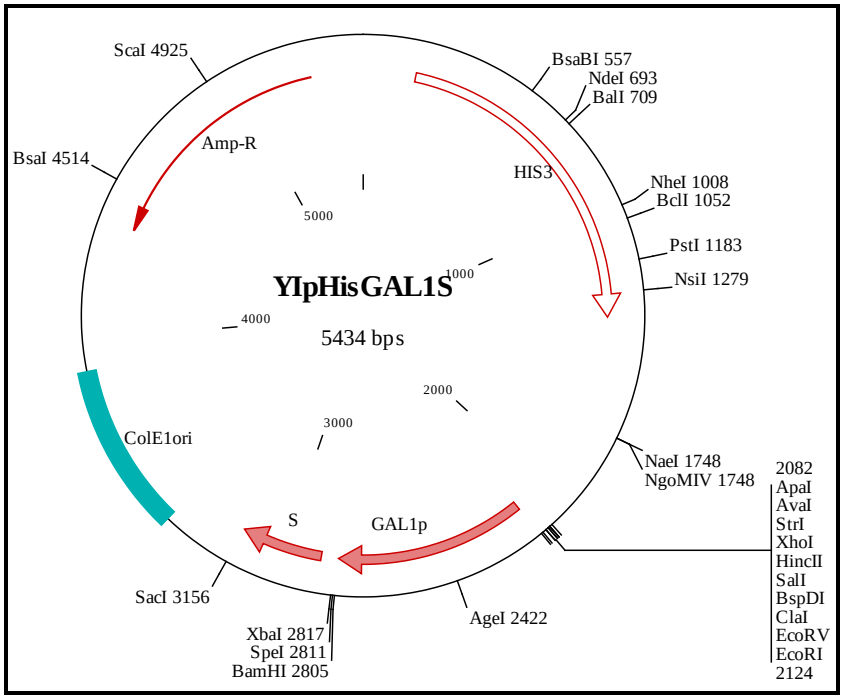


Figure 4.9. Map of YIpHisGAL1S that contains the GAL1 promoter, and which allows integration of the plasmid and its derivatives into the HIS3 locus of the yeast genome. The restriction sites shown are the ones that occur only once in the plasmid.

The plasmid YIpHisGAL1S was digested with *BamH*I, *Sal*I to obtain the 4726 bp vector fragment, YIpHis, devoid of the *GAL1* promoter (Figure 4.10).

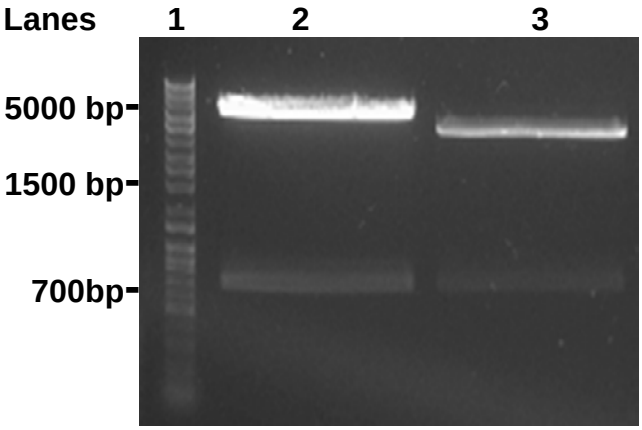


Figure 4.10. The plasmid YIpHisGAL1S (lanes 2, 3) was digested with the restriction enzymes BamHI and SalI. The 4726 bp YIpHis vector fragments, which did not contain the GAL1 promoter fragment (~700bp), were isolated from the agarose gel. Lane1, 2-log DNA ladder.

After ligation of the 4726 bp YIpHis vector fragment to the 593 bp *ADH2* promoter fragment, the plasmid YIpHisADH2S was obtained (Figure 4.11).

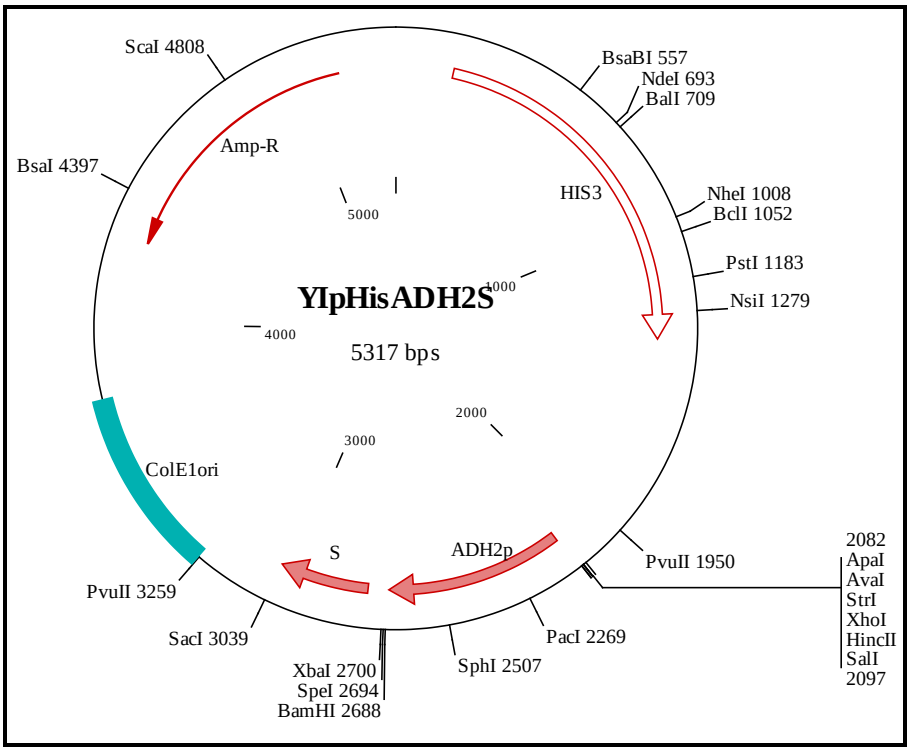


Figure 4.11. The map of the plasmid YIpHisADH2S. It contains the ADH2 promoter (ADH2p) and the SUC2 terminator (S). The plasmid and its derivatives would allow integration into the HIS3 locus of the yeast genome. The map shows restriction sites that would cut the plasmid only once.

The veracity of the plasmid was confirmed by digestion with restriction enzymes, *BamH*I and *Sal*I (Figure 4.12).

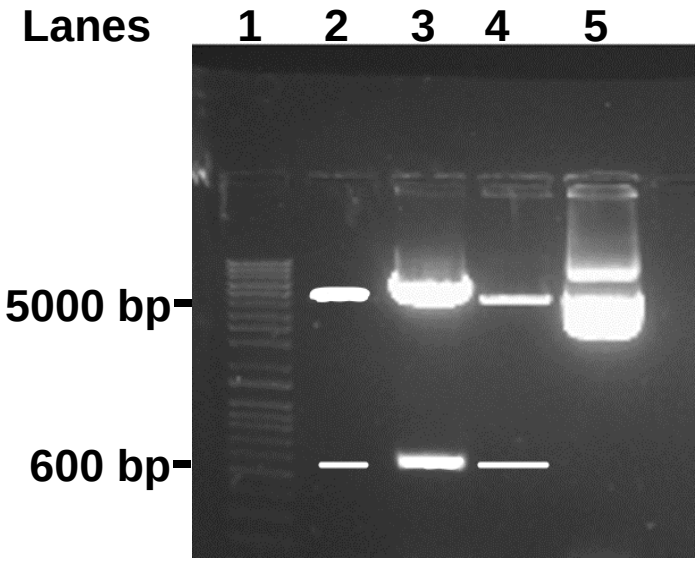


Figure 4.12. An agarose gel that shows the expected fragments (4726 bp and 593 bp) of different clones of the plasmid YIpHisADH2S, after digestion with restriction enzymes BamHI and SalI (lanes 2, 3, 4). Lane 1, 2-log DNA ladder; lane 5, an uncut plasmid.

The YIHisADH2MS plasmid can be used for cloning any gene downstream of the *ADH2* promoter. The gene expression cassettes in the resultant plasmids can then be integrated into the *HIS3* locus on chromosome XV (721946 to 722608bps) of the yeast *S. cerevisiae*.

The *ADE2* and the *HIS3* genes lie on the same chromosome, chromosome XV.

4.5.4 Construction of the yeast integrative plasmid, YIpUraADH2S, that allows integration of a CYP gene expression cassette at the URA3 locus of the yeast genome

The yeast integrative plasmid YIpUraADH2S (the letters in the name of the plasmid has the same meaning as that of the plasmid described in Section 4.2.1, excepting that 'Ura' stands for the functional *URA3* gene) was created via the following steps:

(1) Isolation of vector fragment.

The plasmid YIpUraGAL1S (Figure 4.13) contains the *GAL1* promoter (GAL1). The plasmid was digested with *BamH*I and *Sal*I to obtain the 4654 bp 'YIpUra' fragment (Figure 4.14) which was isolated and used for ligation, as in step (3). Restriction enzyme digestion with *BamH*I, *Sal*I eliminated the *GAL1* promoter to allow its replacement by the *ADH2* promoter.

(2) Isolation of insert fragment.

The 593 bp *ADH2* promoter (ADH2p) insert fragment was isolated from the plasmid pBluKS(+)/ADH2p (Figure 4.3), as in Section 4.4.1.

(3) The vector and insert fragments, from (1) and (2), were ligated to obtain the plasmid YIpUraADH2S (Figure 4.15).

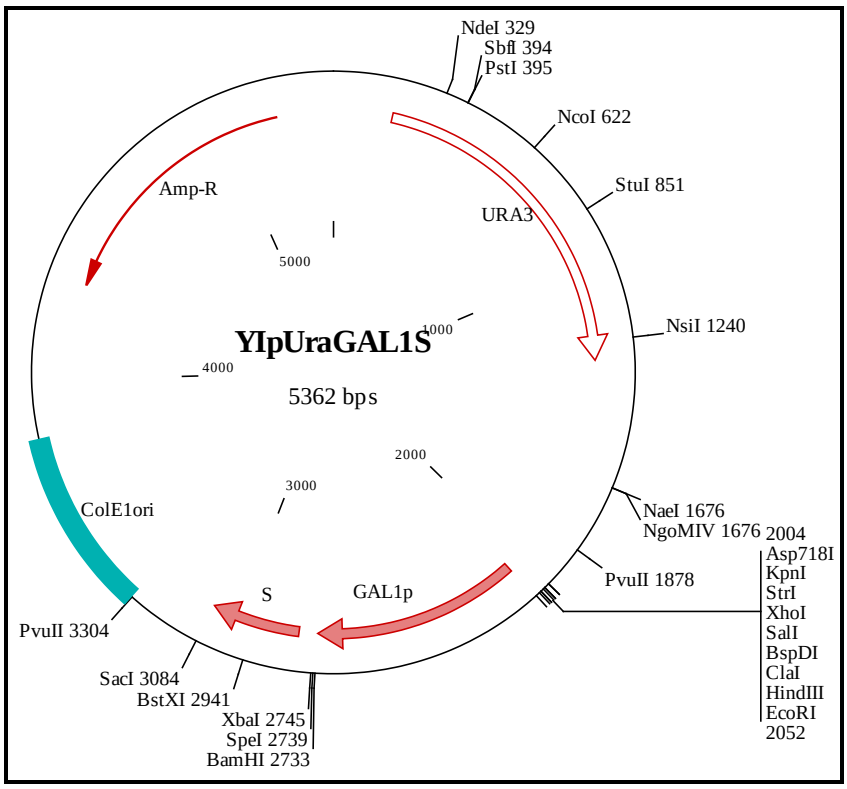


Figure 4.13. Map of YIpUraGAL1S that contains the GAL1 promoter and which allows integration pf the plasmid and its derivatives into the URA3 locus of the yeast genome. The restriction sites shown are the ones that occur only once in the plasmid.

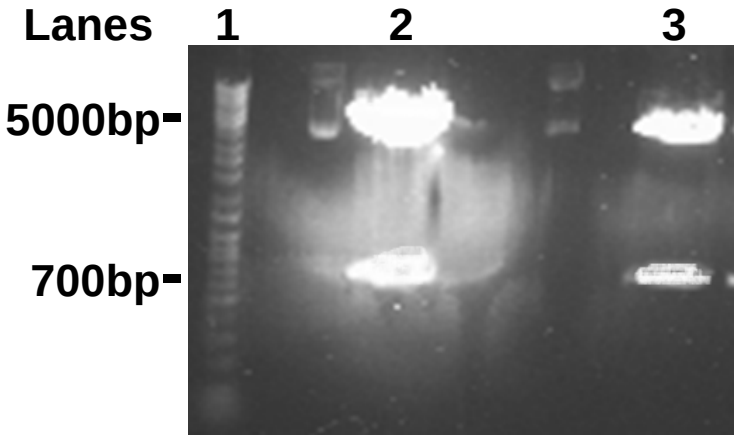


Figure 4.14. The plasmid YIpUraGAL1S (lanes 2, 3) was digested with the restriction enzymes BamHI and SalI. The 4654 bp YIpUra vector fragments, which did not contain the GAL1 promoter fragment (~700bp), were isolated from the agarose gel. Lane 1, 2-log DNA ladder.

The 4654 bp YIpUra vector fragment was ligated to the 593 bp *ADH2* promoter fragment to obtain the plasmid YIpUraADH2S (Figure 4.15).

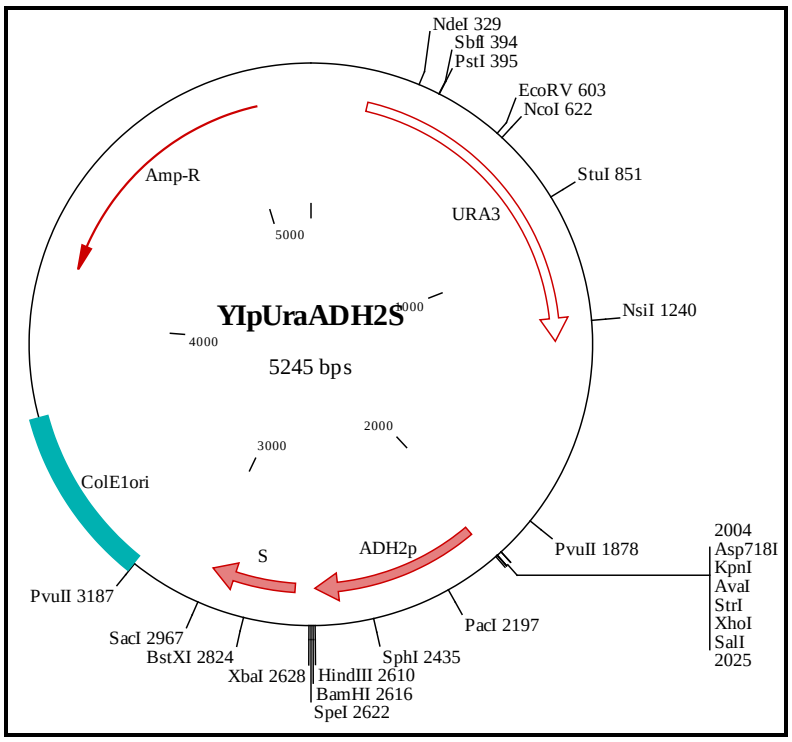


Figure 4.15. The plasmid map of YIpUraADH2S. It contains the *ADH2* promoter (ADH2p) and the *SUC2* terminator (S). The plasmid and its derivatives would allow integration into the URA3 locus of the yeast genome. The map shows restriction sites that would cut the plasmid only once.

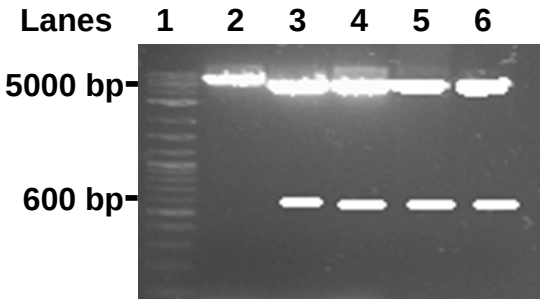


Figure 4.16. An agarose gel that shows the expected DNA fragments (4654, 593 bp) when clones of the plasmid YIUraADH2MS (lanes 3-6) was digested with the restriction enzymes BamHI, SalI. Lane 1, 2-log DNA ladder; lane 2, an uncut plasmid.

The plasmid YIUraADH2S can be used for cloning any gene downstream of the *ADH2* promoter. The gene expression cassettes in the resultant plasmids can then be integrated into the *URA3* locus on chromosome V (116167 to 116970) of the yeast *S. cerevisiae*.

4.6 Construction of yeast strains that bear expression cassettes of the human CYP3A4 gene, chemically synthesized using yeast biased codons, and comparison of CYP3A4 enzyme activities produced by the strains

4.6.1 Construction of a yeast integrative plasmid that would allow stable expression of human CYP3A4 enzyme from the ADE2 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpAdeADH2S/CYP3A4_yc (CYP3A4_yc = gene synthesized using yeast-biased codons which code for the human CYP3A4 protein) was created via the following steps:

(1) After digestion of the vector YIpAdeADH2S (Figure 4.4) with restriction enzymes *BamH*I, *Spe*I, a 6383 bp fragment was isolated. The enzyme digestion eliminates ~20 bp in the multi-cloning site which cannot be seen on the agarose gel (Figure 4.17).

(2) A 1524 bp *BamH*I-*Xba*I CYP3A4_yc gene fragment (i.e. the human *CYP3A4* gene was synthesized with yeast-biased codons based on its protein sequence, NCBI Accession Number NP_059488) was isolated from a pUC57 based plasmid into which the chemically synthesised gene had been cloned at the outset. The made-to-order gene construct was obtained from Genewiz.

(3) The 6383 bp vector and the 1524 bp insert were ligated to obtain the plasmid YIpAdeADH2S/CYP3A4_yc (Figure 4.18).

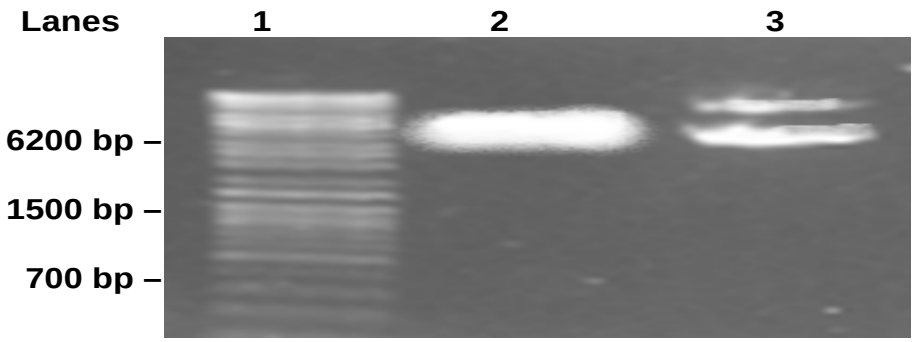


Figure 4.17. An agarose gel shows the digestion of the plasmid YIpAdeADH2S with the restriction enzymes BamHI and SpeI (lane 2). Lane 1, 2-log DNA ladder (3 defined fragments have been labelled on the left); lane 3, uncut plasmid. The 6383 bp vector fragment was isolated from lane 2 of the agarose gel for further ligation.

The *BamH*I-*Spe*I cut 6383 bp vector fragment was ligated with the 1524 bp *BamH*I-*Xba*I CYP34_yc gene fragment to obtain the plasmid YIpAdeADH2S/CYP3A4_yc.

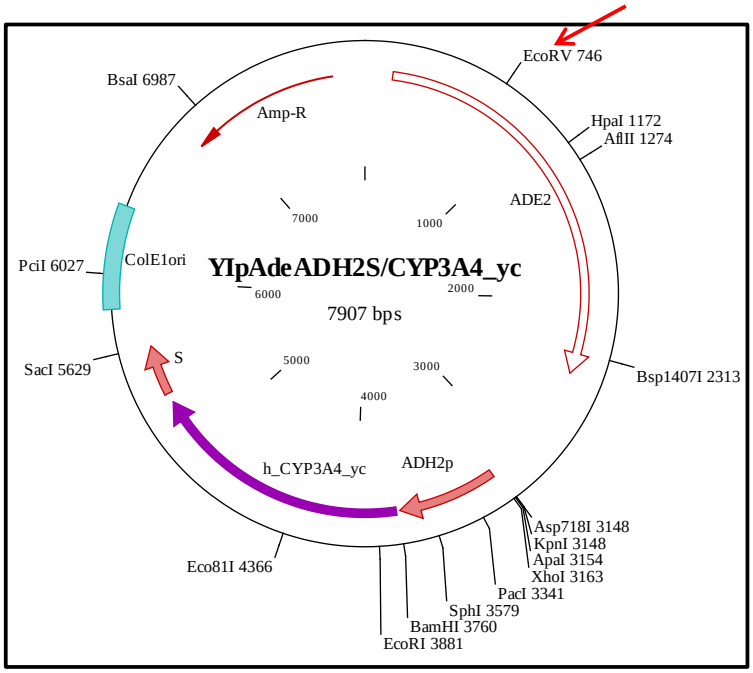


Figure 4.18. Map of plasmid YIpAdeADH2S/CYP3A4_yc that allows integration of a human CYP3A4 gene expression cassette at the ADE2 locus of the yeast genome. The human CYP3A4 gene was synthesized using yeast-biased codons and was named h_CYP3A4_yc. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid was linearized at the *EcoR*V site (indicated by an arrow in Figure 4.18). In order to facilitate homologous recombination, the restriction sites *Hpa*I or *Afl*II (Figure 4.18) could also have been used for linearization.

The newly constructed plasmid was further analysed using a *Sal*I restriction enzyme digest (Figure 4.19).

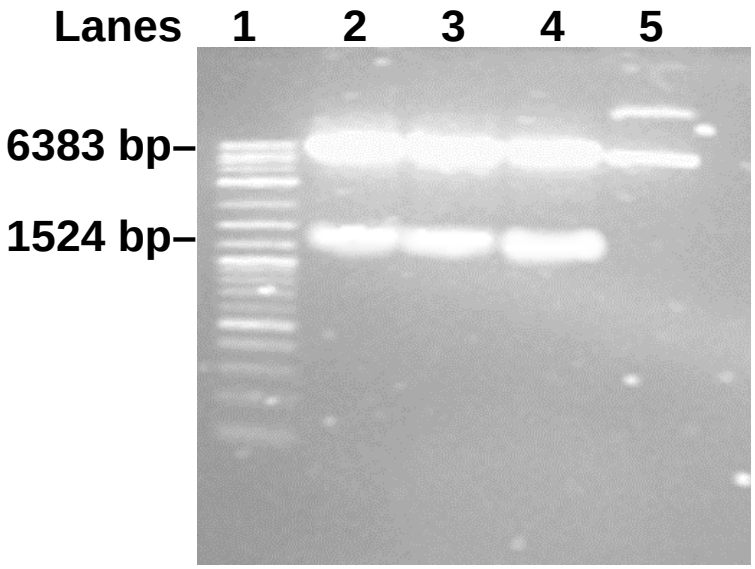


Figure 4.19. The agarose gel that shows the expected DNA fragments (6383, 1524 bp) of YIpAdeADH2pS/CYP3A4_yc when plasmids isolated from three bacterial clones were digested with SalI (lanes 2-4). Lane 1, 2-log DNA ladder; lane 5, uncut plasmid.

The YIAdeADH2S/CYP3A4_yc plasmid can be used for expression of human *CYP3A4* gene, synthesized using yeast-biased codons, driven by the *ADH2* promoter. Upstream of the CYP3A4 gene is a 6A consensus sequence. It has been found, in this laboratory, that the 5'-end 6A consensus sequence provides higher expression of foreign genes in yeast. To allow this to happen, without loss of plasmid, the *CYP3A4_yc* gene expression cassette must be integrated into the yeast genome. YIAdeADH2S/CYP3A4_yc (Figure 4.18)

allows integration into the *ADE2* locus on chromosome XV of yeast cells. The yeast strain that was used for integration of YIAdeADH2S/CYP3A4_yc was YAB79.

The strain YAB79 contained (a) a modified less toxic version of the human reductase, *ΔhRDM* gene under the control of the *ADH2* promoter, and (b) the human cytochrome *b5* gene under the control of the constitutive *GAPDH* promoter. Cells from the YAB79 strain containing an integrated copy of the plasmid YIAdeADH2S/CYP3A4_yc at its *ADE2* locus were named YAB79::3A4_yc(ADE2). Expression from this strain was compared with the levels of expression obtained from the strain YAB79::3A4_yc(263) that expressed human CYP3A4 from the episomal plasmid, pSYE263/CYP3A4_yc (Chapter 3, Section 3.4.2).

4.6.2 Construction of a yeast integrative plasmid that would allow stable expression of human CYP3A4 enzyme from the URA3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIUraADH2pS/CYP3A4_yc was created via the following steps:

(1) After digestion of the vector YIpUraADH2S (Figure 4.15) with restriction enzymes *BamH*I, *Spe*I, a 5239 bp fragment was isolated. The enzyme digestion eliminates ~20 bp in the multi-cloning site which cannot be seen on the agarose gel (Figure 4.20).

(2) The 1524 bp *BamH*I-*Xba*I CYP3A4_yc gene fragment was isolated from a pUC57 based plasmid, as in Section 4.4.1.

(3) The 5239 bp vector and the 1524 bp insert were ligated to obtain the plasmid YIpUraADH2S/CYP3A4_yc (Figure 4.21).

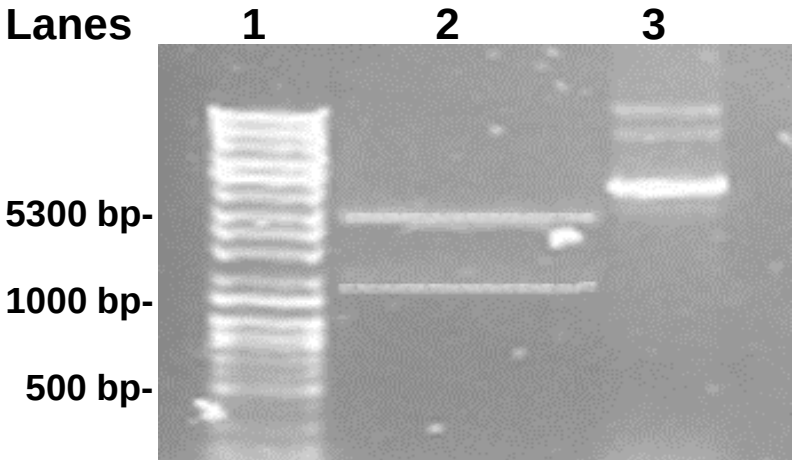


Figure 4.20. An agarose gel showing the digestion of the plasmid YIpUraADH2S with the restriction enzymes BamHI and SpeI (lane 3). Lane 1, 2-log DNA ladder, showing 3 defined DNA fragments; lane 2, uncut plasmid. The 5239 bp vector fragment was isolated from lane 2 of the agarose gel for further ligation.

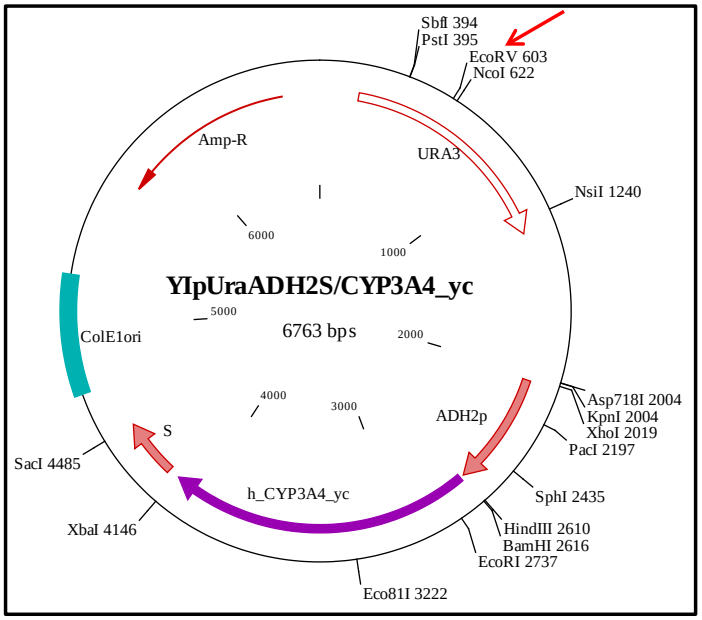


Figure 4.21. Map of plasmid YIpUraADH2S/CYP3A4_yc that allows integration of a human CYP3A4 gene expression cassette at the URA3 locus of the yeast genome. The human CYP3A4 gene, synthesized using yeast-biased codons, is referred to as h_CYP3A4_yc. The map shows restriction sites that cut the plasmid only once.

For integration into the yeast strain YAB79, via homologous recombination, the plasmid YIpUraADH2S/CYP3A4_yc was linearized at the *EcoR*V site (indicated by an arrow in Figure 4.21). In order to facilitate homologous recombination, the restriction site *Nco*I (Figure 4.21) could also have been used for linearization.

The veracity of the plasmid was confirmed by digestion with the restriction enzymes *BamH*I, *Xba*I (Figure 4.22) and it showed expected sizes of fragments.

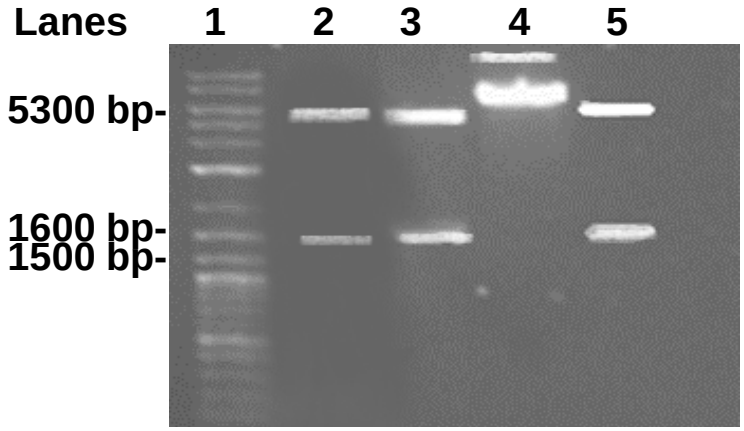


Figure 4.22. The agarose gel that shows the expected DNA fragments (5239, 1524 bp) of YIpUraADH2pS/CYP3A4_yc when plasmids isolated from three bacterial clones were digested with SalI (lanes 2, 3, 5). Lane 1, 2-log DNA ladder; lane 4, uncut plasmid.

The YIpUraADH2S/CYP3A4_yc plasmid can be used for expression of *CYP3A4_yc* gene driven by the *ADH2* promoter. To allow this, the *CYP3A4_yc* gene expression cassette must be integrated into the *URA3* locus on chromosome V of the yeast genome.

The resultant strain YAB79::3A4_yc(URA3) was used to analyse levels of expression of human CYP3A4 enzyme and compared with the levels of expression obtained from the strain YAB79::3A4_yc(263) expressing CYP3A4 from the episomal plasmid, pSYE263/CYP3A4_yc (Chapter 3, Section 3.4.2).

4.6.3 Construction of a yeast integrative plasmid that would allow stable expression of human CYP3A4 enzyme from the HIS3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpHisADH2S/CYP3A4_yc was created via the following steps:

(1) After digestion of the vector YIpHisADH2S (Figure 4.11) with restriction enzymes *BamH*I, *Spe*I, a 5311 bp fragment was isolated. The enzyme digestion eliminates a small ~20 bp in the multi-cloning site which cannot be seen on the agarose gel (Figure 4.23).

(2) The 1524 bp *BamH*I-*Xba*I CYP3A4_yc gene fragment was once again isolated from a pUC57 based plasmid, as in Section 4.4.1.

(3) The 5311 bp vector and the 1524 bp insert were ligated to obtain the plasmid YIpHisADH2S/CYP3A4_yc (Figure 4.24).

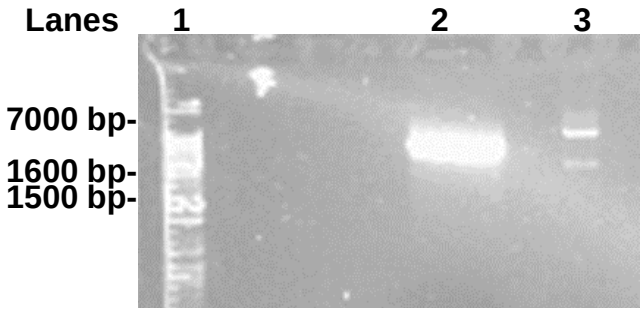


Figure 4.23. An agarose gel showing the digestion of the plasmid YIpHisADH2S with the enzymes BamHI and SpeI (lane 2) and the plasmid pUC57/CYP3A4_yc with the enzymes BamHI and XbaI (lane 3). Lane 1, 2-log DNA ladder. The 5311 bp vector and the 1524 bp insert fragments were isolated from lanes 2 and 3 of the agarose gel for ligation to obtain YIpHisADH2S/CYP3A4_yc (Figure 4.24).

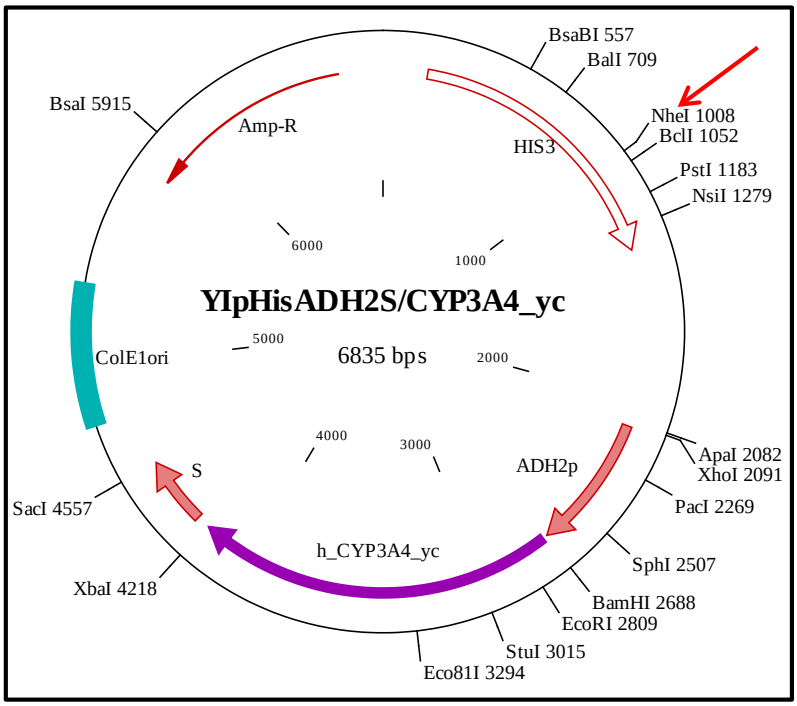


Figure 4.24. Map of plasmid YIpHisADH2S/CYP3A4_yc that allows integration of a human CYP3A4 gene expression cassette at the HIS3 locus of the yeast genome. The human CYP3A4_yc gene was synthesized using yeast-biased codons. The map shows restriction sites that cut the plasmid only once.

For integration into the yeast strain YAB79, via homologous recombination, the plasmid YIpHisADH2S/CYP3A4_yc was linearized at the *Nhe*I site (indicated by an arrow in Figure 4.24). In order to facilitate efficient homologous recombination, alternatively the restriction sites *Bal*I or *Bcl*I (Figure 4.24) could also have been used for linearization.

In order to confirm that the plasmid YIpHisADH2S/CYP3A4_yc was constructed correctly, plasmid DNA obtained from bacterial clones via alkaline lysis (see Chapter 2, Section 2.4.1.8) was digested with the restriction enzyme *Pvu*II (Figure 4.25). The plasmid showed expected sizes of fragments.

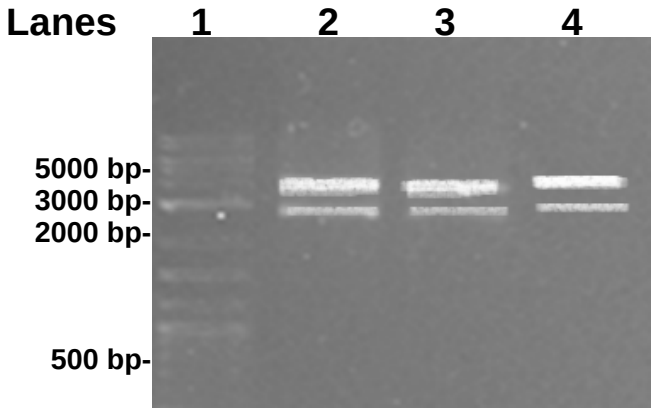


Figure 4.25. The agarose gel that shows the expected DNA fragments (4008, 2821 bp) of YIpHisADH2pS/CYP3A4_yc when plasmid DNA isolated from three bacterial clones were digested with the restriction enzyme PvuII (lanes 2, 3, 4). Lane 1, 2-log DNA ladder.

The plasmid YIpHisADH2pS/CYP3A4_yc can be used for expression of the *CYP3A4_yc* gene driven by the *ADH2* promoter. To allow this, the *CYP3A4_yc* gene expression cassette must be integrated into the *HIS3* locus on chromosome XV.

The resultant strain YAB79::3A4_yc(HIS3) was used to analyse levels of expression of human CYP3A4 enzyme and compared with the levels of expression obtained from the strain YAB79::3A4_yc(263) which expresses CYP3A4 from the episomal plasmid, pSYE263/CYP3A4_yc (Chapter 3, Section 3.4.2).

4.6.4 Construction of yeast strains that contain a copy of the CYP3A4_yc gene expression cassette integrated into three different chromosomal loci of the yeast strain YAB79

Three yeast strains were generated that contained a single copy of the *CYP3A4_yc* gene expression cassette. They were obtained by individually integrating the plasmids that bear the *CYP3A4_yc* expression cassette, in the yeast strain YAB79.

The strain YAB79 contains a modified version of the human P450 reductase, *hRD*, which is referred to as *ΔhRDM*. The modified gene was cloned downstream of the *ADH2* promoter and its gene expression cassette was integrated at the *LEU2* chromosomal locus of the strain BC300 which was originally derived from the commercially available strain W303-1A (ATCC # 208352). After integration of the *ΔhRDM* gene expression cassette, the resultant strain was named YY7. Cytochrome *b5* is the substrate of the cytochrome *b5* reductase which is an alternate source of electrons for activation of cytochrome P450 (CYP) enzymes. A human cytochrome *b5* gene was cloned downstream of the constitutive *GAPDH* promoter that would allow constant expression of cytochrome *b5* throughout the growth of cells from time zero (t= 0). The human cytochrome *b5* gene expression cassette was integrated at the *TRP1* chromosomal locus of YY7 to obtain the strain YAB79 (BC300::ΔhRDM/LEU2^{+}, b5/TRP1^{+}; Figure 4.26).

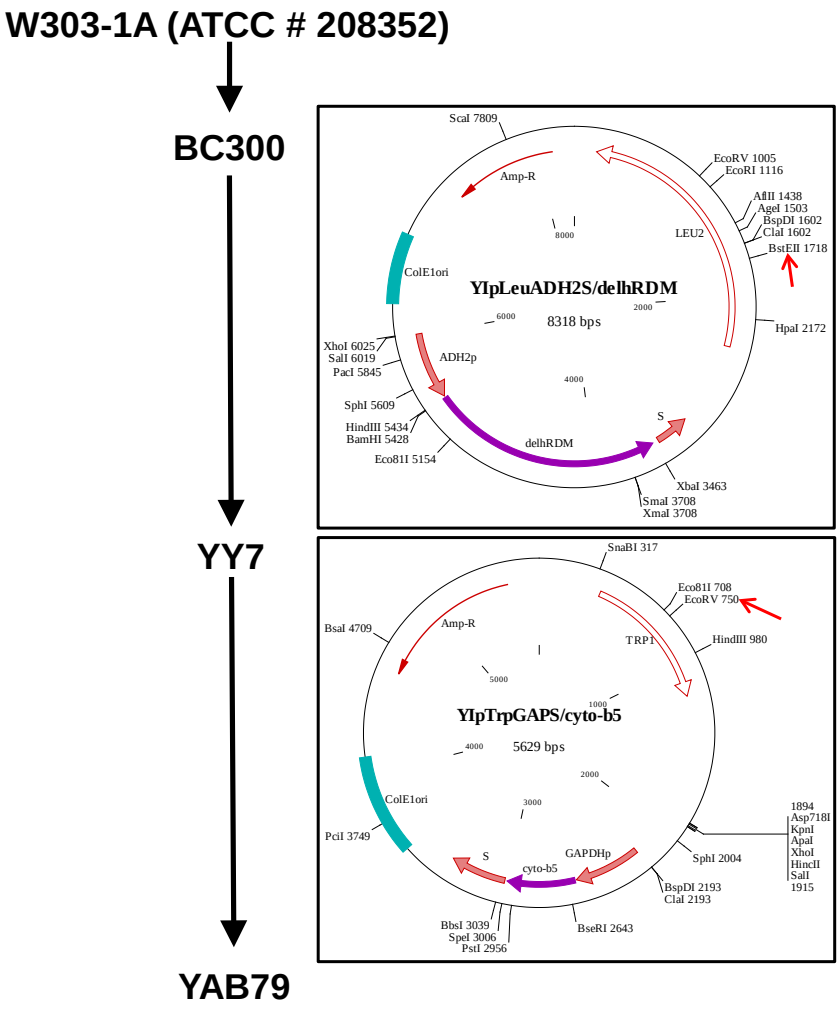


Figure 4.26. Construction of the yeast strain YAB79 from BC300. (a) Plasmid YIpLeuADH2S/ΔhRDM was integrated at the LEU2 locus of strain BC300 to create the strain YY7. (b) Plasmid YIpTrpGAPS/cyto-b5 was integrated at the TRP1 locus of the strain YY7 to create the strain YAB79.

The *CYP3A4_yc* gene encoding plasmids used for integration in the strain YAB79 were:

(1) YIpAdeADH2S/CYP3A4_yc,

(2) YIpUraADH2S/CYP3A4_yc, and

(3) YIpHisADH2S/CYP3A4_yc.

After integration, the strains were named:

(a) YAB79::3A4_yc(ADE2),

(b) YAB79::3A4_yc(URA3), and

(c) YAB79::3A4_yc(HIS3).

4.6.5 Comparison of activities of enzyme expressed in yeast cells from (a) chromosomal integrants and (b) an episomal plasmid bearing CYP3A4_yc gene

The activities of CYP3A4 enzyme expressed from different chromosomal loci, *ADE2*, *HIS3* and *URA3*, were compared. The enzyme expressed from a strain containing *CYP3A4* gene-bearing episomal plasmid [YAB79::3A4_yc(2µ)] was used as a positive control whereas any enzyme activity from cells containing an empty integrated plasmid (*ADE2*, *HIS3* or *URA3*) was used as a negative control (Figure 4.27).

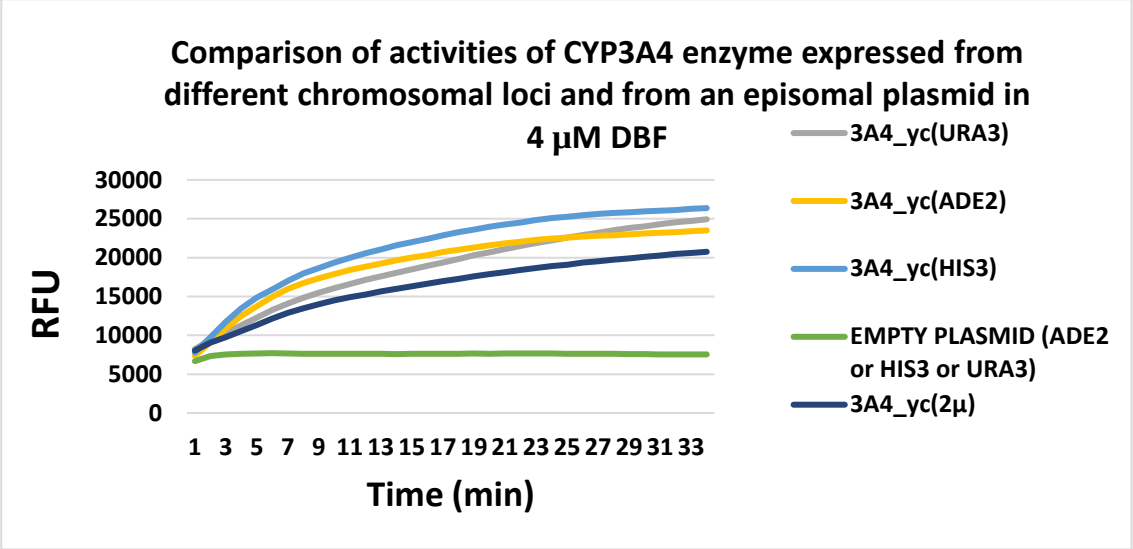


Figure 4.27. The graph shows the activities of CYP3A4 enzyme expressed from a single copy of the CYP3A4_yc gene expression cassette, integrated at the URA3, ADE2 or HIS3 chromosomal locus, in the yeast strain YAB79. Activities are compared with the CYP3A4 enzyme expressed from an episomal plasmid. The graphs represent the average of results obtained from three independent experiments.

It appears from Figure 4.27 that the CYP3A4 enzyme expressed from the *HIS3* locus has the best activity, better than that produced from the *URA3* or the *ADE2* locus of the strain YAB79. The kinetic analysis of the CYP3A4 enzyme, co-expressed with cytochrome b5 and cytochrome P450 reductase (CPR; ΔhRDM), and expressed from different strains were performed, using DBF as a substrate. The intact yeast cells bearing the *CYP3A4_yc* gene integrated in chromosomal loci (*ADE2*, *HIS3* and *URA3*), an episomal plasmid bearing the *CYP3A4_yc* gene and the control empty plasmid yeast strain were grown as described in Section 4.1.1 and the kinetics of the enzymes produced were compared, as described in Section 4.1.2, to measure the formation of the product, fluorescein. The procedures were carried out in three independent experiments.

The activities obtained at 30 min (in Figure 4.27) were re-plotted as bars to show clearly that enzyme expressed from the *HIS3* locus has the best activity (Figure 4.28).

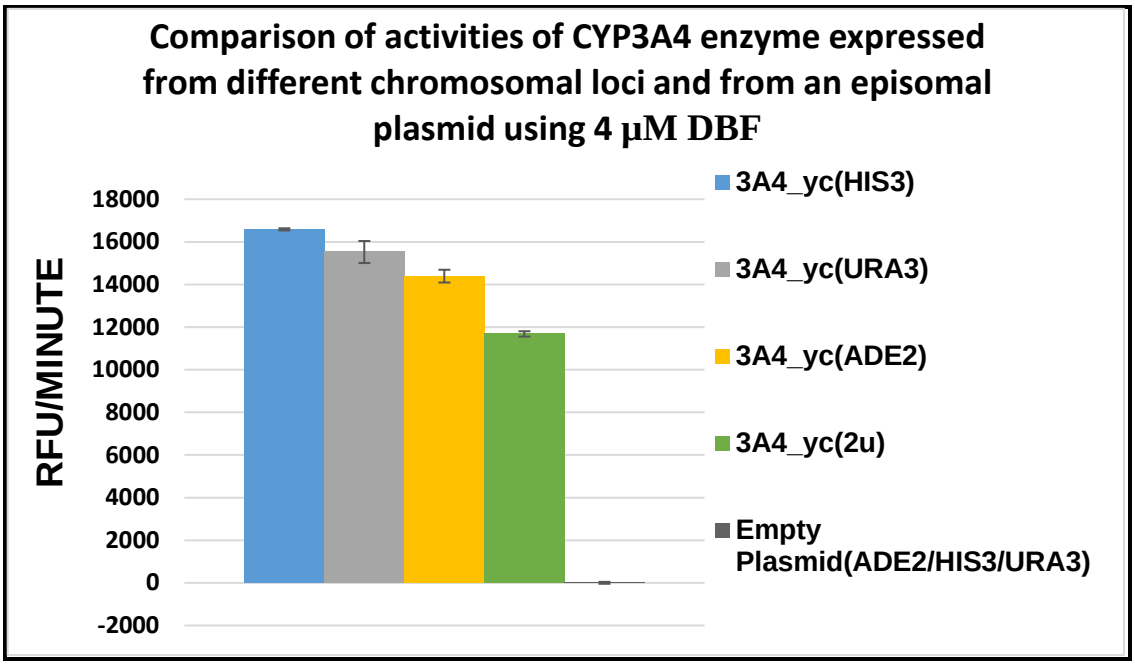


Figure 4.28. The bar plot shows the activities of CYP3A4 enzyme expressed from a single copy of the CYP3A4_yc gene expression cassette integrated at the ADE2, HIS3 or URA3 locus of the yeast strain YAB79 and compared with that obtained from an episomal 2-micron (2µ) plasmid. The bars mirror the readout, obtained at 30 min, shown in Figure 4.27. The data represent mean ± S.D. of three independent experiments.

Figure 4.28 shows that the CYP3A4 enzyme expressed from the *HIS3* locus has better activity than the one from the *URA3* locus while the enzyme produced from the *URA3* locus was better than that from the *ADE2* locus. The latter activity was better than that obtained from the episomal plasmid. Further corroboration of these results is revealed in Chapter 5 of this study.

The graphs in Figures 4.27 and 4.28 would suggest that the amounts of CYP3A4 expressed depend on the genetic locus and the chromosome in which the *CYP3A4_yc* gene expression cassette is integrated. The results also show that the amounts of CYP3A4 expressed in cells containing a single copy of *CYP3A4_yc* gene is higher than in cells which express CYP3A4 enzyme from an episomal 2-micron (2µ) plasmid. *CYP3A4_yc*

gene, borne on an episomal plasmid should, in theory, provide multiple copies per cell and, therefore, should have yielded more CYP3A4 than in cells which contain a single copy of the gene. Our results suggest that CYP3A4 expression occurs more stably from a chromosomal locus than from an extra-chromosomal 2µ-plasmid.

4.7 Construction of yeast strains bearing expression cassettes of the human CYP1A2 gene, chemically synthesized using yeast biased codons, and comparison of CYP1A2 enzyme activities produced by the strains

4.7.1 Construction of a yeast integrative plasmid that would allow stable expression of human CYP1A2 enzyme from the HIS3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpHisADH2S/CYP1A2_yc (CYP1A2_yc = gene coding for human CYP1A2, synthesized using yeast-biased codons) was created via the following steps:

(1) After digestion of the vector YIpHisADH2S (Figure 4.11) with restriction enzymes *BamH*I, *Xba*I, a 5305 bp fragment was isolated (Figure 4.29). This eliminated ~20 bp in the multi-cloning site.

(2) A 1575 bp *BamH*I-*Xba*I *CYP1A2_yc* gene fragment was isolated from a pUC57 based plasmid into which the chemically synthesised gene had been cloned. The tailor-made gene construct was obtained from Genewiz. *CYP1A2_yc* gene was synthesized with yeast-biased codons using the human CYP1A2 protein as a template (Accession Number of protein sequence, NM_000761).

(3) The 5305 bp vector and the 1575 bp insert were ligated to obtain the plasmid YIpHisADH2S/CYP1A2_yc (Figure 4.29).

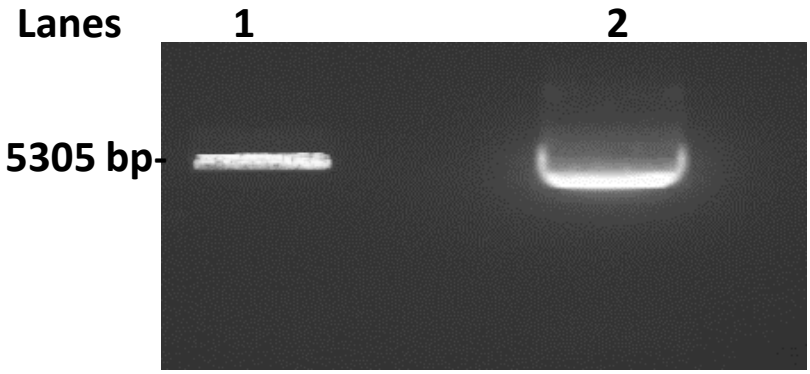


Figure 4.29. An agarose gel showing the digestion of the plasmid YIpHisADH2S with the restriction enzymes BamHI and Xbal (lane 1). Lane 2, uncut plasmid.

The 5305 bp vector fragment was isolated from lane 1 of the agarose gel (Figure 4.29) for ligation with the 1575 bp *CYP1A2_yc* gene fragment to obtain the plasmid YIpHisADH2S/CYP1A2_yc (Figure 4.30).

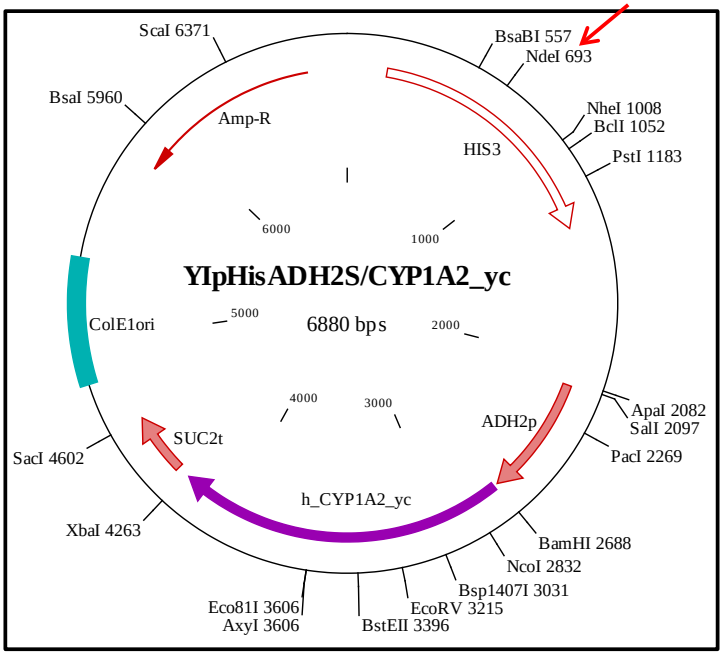


Figure 4.30. Map of plasmid YIpHisADH2S/CYP1A2_yc that allows integration of a human CYP1A2 gene expression cassette at the HIS3 locus of the yeast genome. The human CYP1A2 gene was synthesized using yeast-biased codons and was named h_CYP1A2_yc. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpHisADH2S/CYP1A2_yc was linearized at the *Nde*I site (indicated by an arrow in Figure 4.30). In order to facilitate homologous recombination, the restriction sites *Nhe*I or *Bal*I (Figure 4.30) could also have been used for linearization.

The newly constructed plasmid YIpHisADH2S/CYP1A2_yc was further analysed via digestion with *Xho*I and *Bam*I + *Sac*I restriction enzymes (Figure 4.31). The agarose gels showed the expected sizes of DNA fragments.

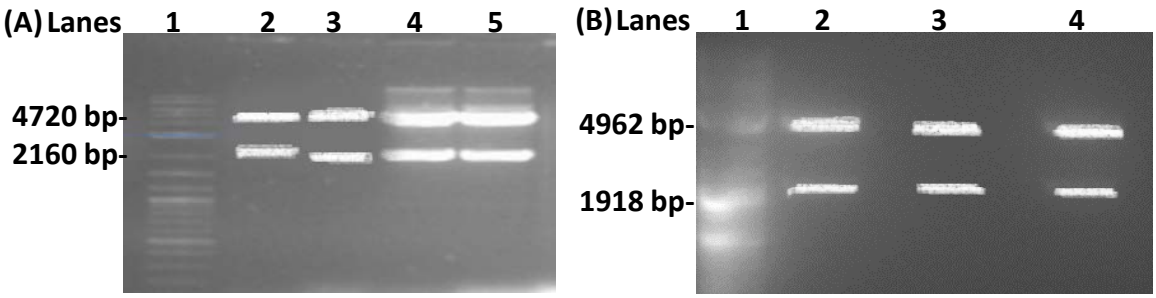


Figure 4.31. Agarose gels (A, B) that show the expected DNA fragments of YIpHisADH2S/CYP1A2_yc when plasmids isolated from four (A) or three (B) bacterial clones were digested with XhoI (lanes 2-5; A) and BamHI-SacI (lanes 2-4; B). Lane 1 (A & B), 2-log DNA ladder.

The YIpHisADH2S/CYP1A2_yc plasmid can be used for expression of the *CYP1A2_yc* gene driven by the *ADH2* promoter. To allow this, the *CYP1A2_yc* gene expression cassette was integrated into the *HIS3* locus on chromosome XV.

4.7.2 Construction of a yeast integrative plasmid that would allow stable expression of human CYP1A2 enzyme from the URA3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpUraADH2S/CYP1A2_yc was created via the following steps:

(1) After digestion of the vector YIpUraADH2S (Figure 4.15) with restriction enzymes *BamH*I, *Xba*I, a 5233 bp fragment was isolated. The enzyme digestion eliminates 12 bp in the multi-cloning site which cannot be seen on the agarose gel (Figure 4.32).

(2) The 1575 bp *BamH*I-*Xba*I CYP1A2_yc gene fragment was isolated from a pUC57 based plasmid, as in Section 4.5.1.

(3) The 5233 bp vector and the 1575 bp insert were ligated to obtain the plasmid YIpUraADH2S/CYP1A2_yc (Figure 4.33).

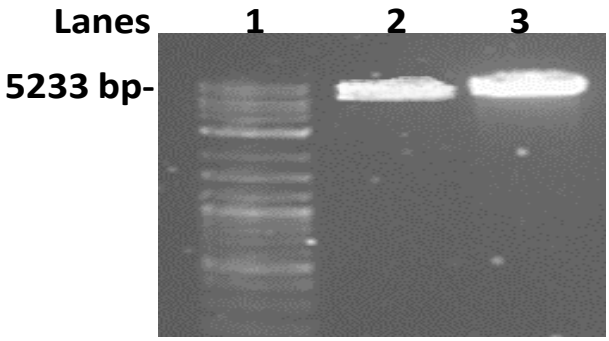


Figure 4.32. An agarose gel showing the digestion of the plasmid YIpUraADH2S with the restriction enzymes BamHI and Xbal (lanes 2, 3). Lane 1, 2-log DNA ladder.

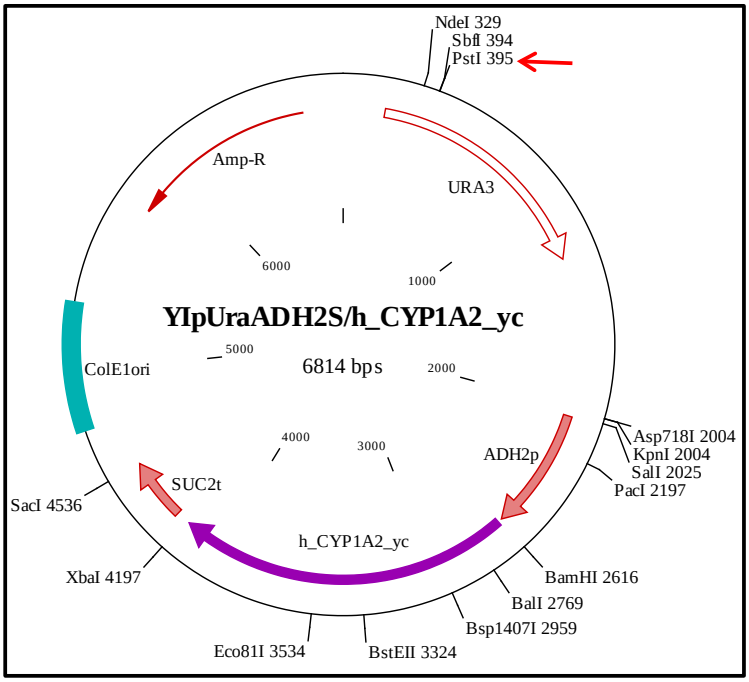


Figure 4.33. Map of plasmid YIpUraADH2S/CYP1A2_yc that allows integration of a human CYP1A2 gene expression cassette at the URA3 locus of the yeast genome. The human CYP1A2 gene was synthesized using yeast-biased codons and was named h_CYP1A2_yc. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpUraADH2S/CYP1A2_yc was linearized at the *Pst*I site (indicated by an arrow in Figure 4.33).

The newly constructed plasmid YIpUraADH2S/CYP1A2_yc was further analysed via digestion with *Xho*I and *Bam*I + *Sac*I restriction enzymes (Figure 4.34). The agarose gels showed the expected sizes of DNA fragments.

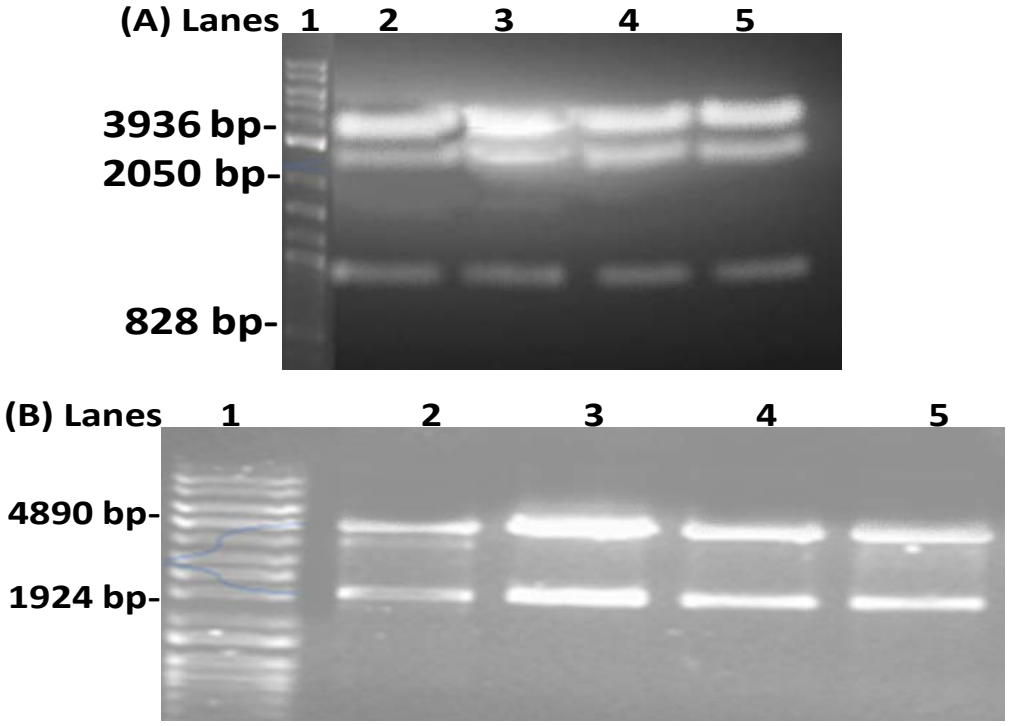


Figure 4.34. Agarose gels (A, B) that show the expected DNA fragments of YIpUraADH2S/CYP1A2_yc when plasmids isolated from four bacterial clones were digested with PvuII (lanes 2-5; A) and BamHI-SacI (lanes 2-5; B). Lane 1 (A & B), 2-log DNA ladder.

The plasmid YIpUraADH2S/CYP1A2_yc can be used for expression of the *CYP1A2_yc* gene driven by the *ADH2* promoter. To allow this, the *CYP1A2_yc* gene expression cassette was integrated into the *URA3* locus on chromosome V.

4.7.3 Construction of yeast strains that contain a copy of the CYP1A2_yc gene expression cassette integrated into two different chromosomal loci of the yeast strains YY7 and YAB79

Four yeast strains were generated that contained a single copy of the *CYP1A2_yc* gene expression cassette. They were obtained by individually integrating the plasmids that bear the *CYP1A2_yc* expression cassette, in the yeast strains YY7 and YAB79.

The genesis of the strain YAB79 (BC300::ΔhRDM/LEU2$^+$, b5/TRP1$^+$; Figure 4.26) has been explained in Section 4.4.4. It expresses ΔhRDM, a modified version of the human CYP450 reductase, hRD, the gene being expressed from the *ADH2* promoter. It also co-expresses human cytochrome b5 from the *GAPDH* promoter. The strain YY7 (BC300::ΔhRDM/LEU2$^+$) expresses only ΔhRDM but not cytochrome b5.

The *CYP1A2_yc* gene encoding plasmids used for integration in the strains YY7 and YAB79 (see Section 4.4.4) were:

(1) YIpUraADH2S/CYP1A2_yc, and

(2) YIpHisADH2S/CYP1A2_yc.

After integration, the four strains were named:

(a) YY7::1A2_yc(URA3), YAB79::1A2_yc(URA3), and

(b) YY7::1A2_yc(HIS3), YAB79::1A2_yc(HIS3).

4.7.4 Comparison of activities of CYP1A2 enzyme expressed in yeast cells from (a) chromosomal integrants of CYP1A2_yc gene expression cassettes and (b) an episomal plasmid bearing CYP1A2_yc gene

The activities of CYP1A2 enzyme expressed from two different chromosomal loci, *URA3* and *HIS3* were compared. The enzyme expressed from a strain containing *CYP1A2* gene-bearing episomal plasmid [YAB79::1A2_yc(2µ)] was used as a positive control whereas any enzyme activity from cells containing an empty integrated plasmid (*URA3* or *HIS3*) was used as a negative control (Figure 4.35). The comparative kinectic analysis of CY1A2_yc co-expressed with and without cytochrome b5 with cytochrome P450 reductase (CPR) using CEC as a substrate. The intact yeast cells bearing the CYP1A2 integrated in chromosomal loci (HIS3 and URA3), the episomal CYP3A4_yc and the control empty plasmid yeast strain were grown as described in section 4.1.1 and the comparative enzyme kinetic assays were measure as described in section 4.1.2 to measure the CHC formation. This procedures were carried out in three different experiments

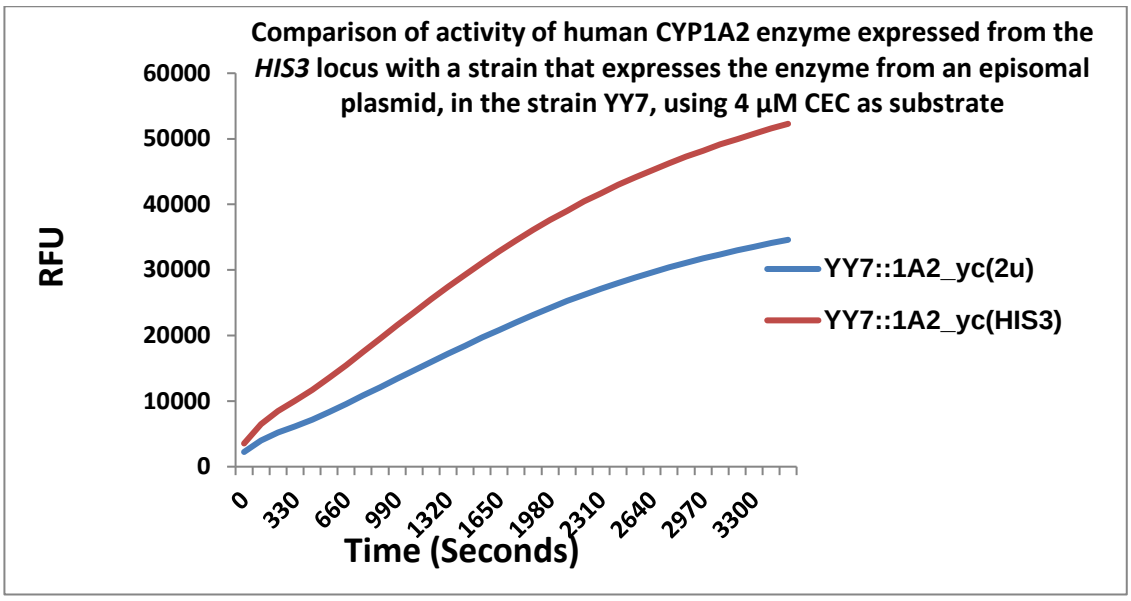


Figure 4.35. The graph shows the activity of CYP1A2 enzyme expressed from a single copy of the CYP1A2_yc gene expression cassette, integrated at the HIS3 chromosomal locus, in the yeast strain YY7 which lacks cytochrome b5. Activity is compared with the CYP1A2 enzyme expressed in YY7 from an episomal plasmid. The graphs represent the average of results obtained from three independent experiments.

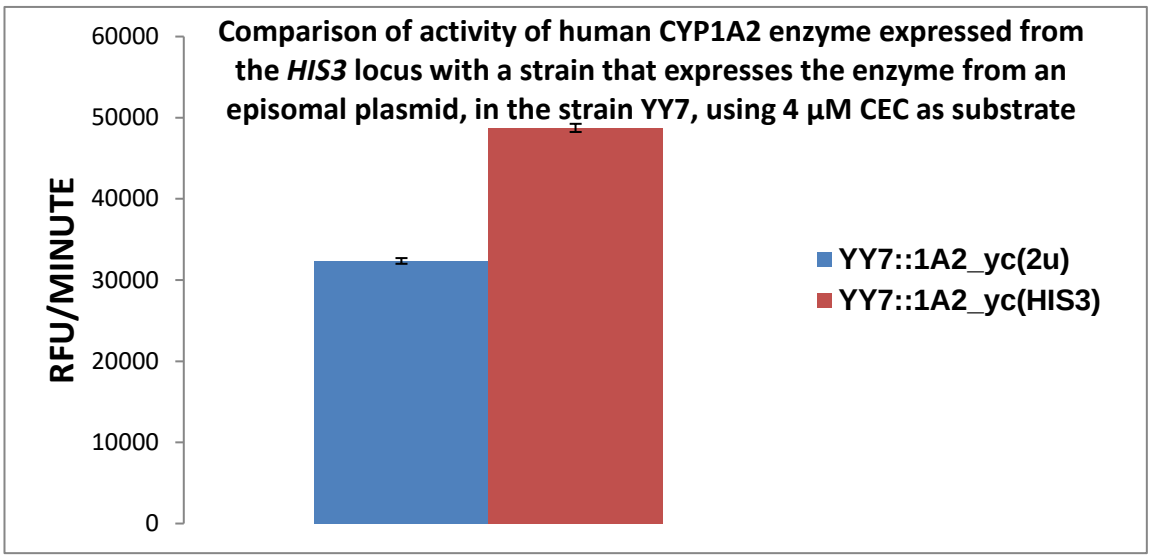


Figure 4.36. The bar plot shows the activity of CYP1A2 enzyme expressed from a single copy of the CYP1A2_yc gene expression cassette integrated at the HIS3 locus of the yeast strain YY7, which lacks cytochrome b5, and compared with that obtained from an epiosmal 2-micron (2µ) plasmid. The data represent mean ± S.D. of three independent experiments.

These results once again show that the amounts of a human CYP, in this case CYP1A2, expressed in cells which contain a single copy of a *CYP_yc* gene, integrated at a particular chromosomal locus, is higher than in cells which express CYP1A2 enzyme from an episomal 2µ-plasmid. As mentioned before, the *CYP1A2_yc* gene, borne on an episomal plasmid should, in theory, provide multiple copies per cell and, therefore, should have yielded more CYP1A2 than in cells which contain a single copy of the gene. This definitely suggests that a human CYP expression, in yeast, occurs more stably from a chromosomal locus than from an extra-chromosomal multi-copy plasmid.

4.7.5 Comparison of activities of CYP1A2 enzyme expressed in YY7 and YAB79

The levels of expression of *CYP1A2_yc* gene, in YY7 (which does not contain the cytochrome *b5* gene) and in YAB79 which expresses the human cytochrome b5 protein were then compared. The results, in Figure 4.37, show that the presence of cytochrome b5 within cells dramatically increases CYP1A2 activity, i.e. more than 2-fold.

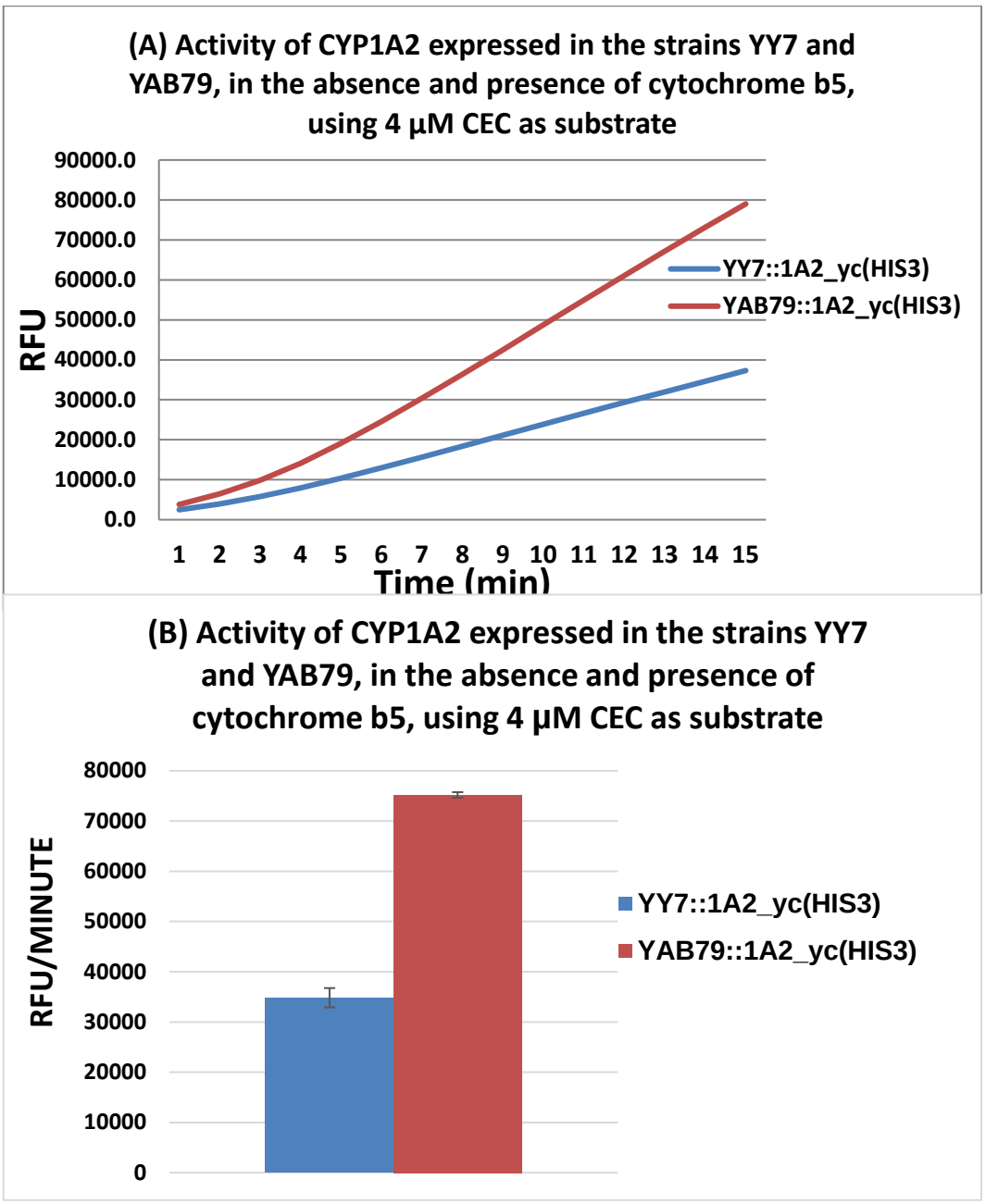


Figure 4.37. The graph (A) shows the activity of CYP1A2 enzyme expressed from a single copy of the CYP1A2_yc gene expression cassette, integrated at the HIS3 chromosomal locus, in the yeast strain YY7 which lacks cytochrome b5. Activity is compared with the CYP1A2 enzyme expressed in the strain YAB79 which expresses cytochrome b5. The graphs represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

4.7.6 Comparison of activities of CYP1A2 enzyme expressed in the yeast strain YAB79, from two different loci, HIS3 and URA3

Activities of human CYP1A2 enzyme expressed from two different chromosomal loci, *HIS3* and *URA3*, were then compared. The results are depicted in Figure 4.38 and show that chromosomal location of the *CYP1A2_yc* gene at the *HIS3* locus has huge effect (greater than 4-fold) on CYP1A2 levels of expression.

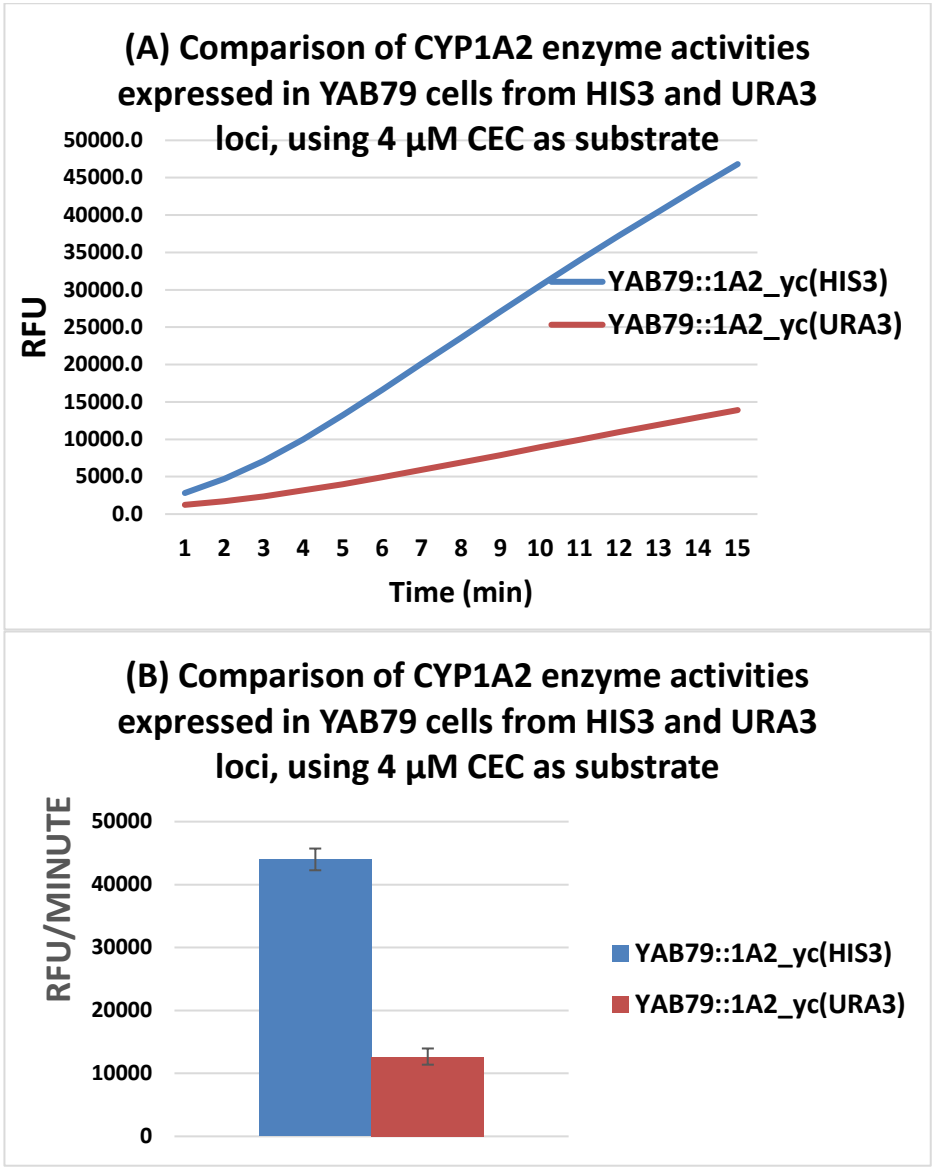


Figure 4.38. The graph (A) compares the CYP1A2 enzyme activity produced by a copy of the CYP1A2_yc gene expression cassette, integrated at the HIS3 and URA3 chromosomal loci, in the yeast strain YAB79 which co-expresses cytochrome b5. The graphs represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

The amounts of CYP1A2 expressed from an integrated copy of the *CYP1A2_yc* expression cassette at the *URA3* locus were then compared with the amounts produced from an extra-chromosomal episomal plasmid (Figure 4.39).

4.7.7 Comparison of activities of CYP1A2 enzyme expressed from (a) the URA3 chromosomal locus and (b) an episomal plasmid in the yeast strain YAB79

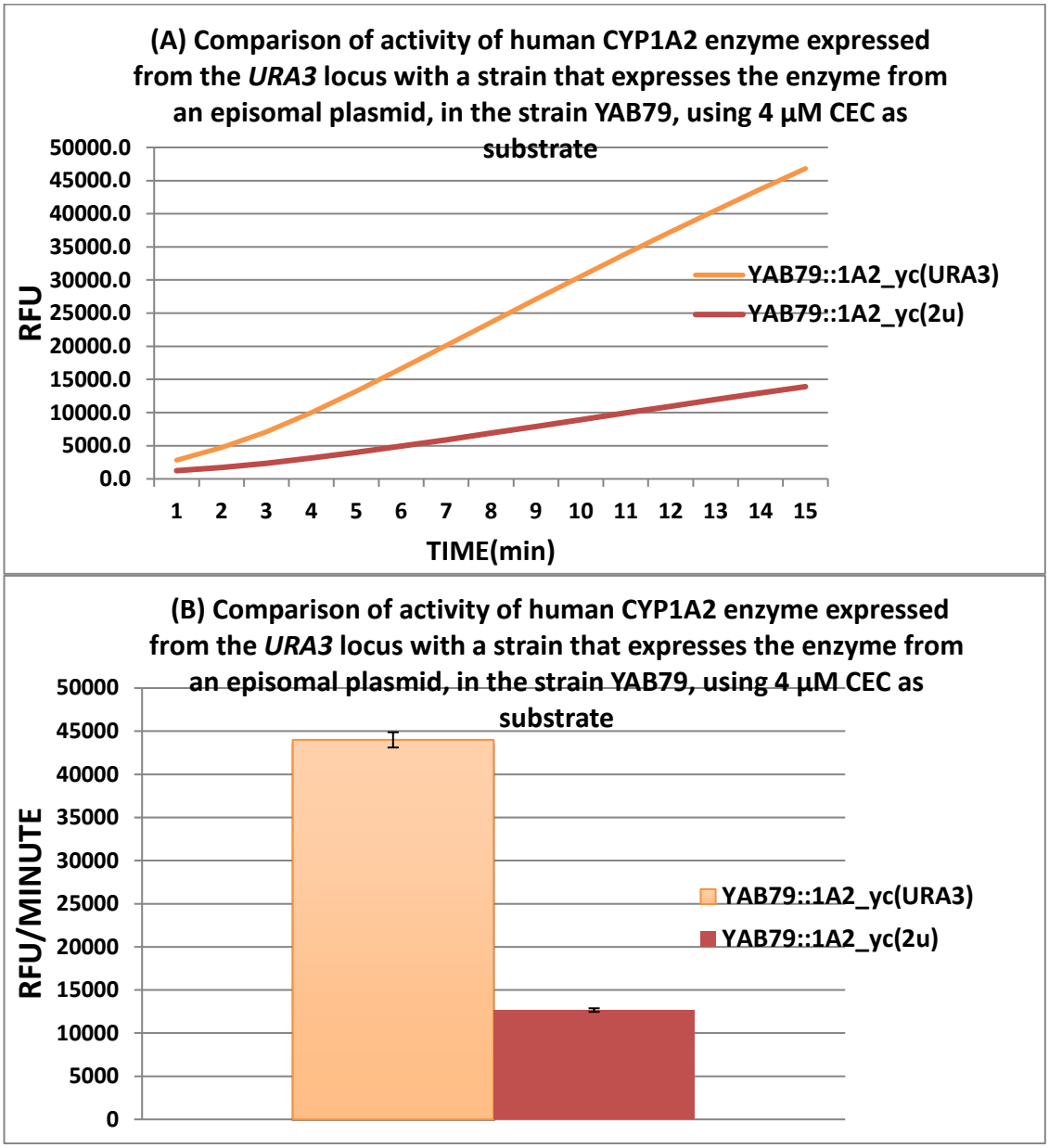


Figure 4.39. The graph (A) compares the CYP1A2 enzyme activity produced by a copy of the CYP1A2_yc gene expression cassette, integrated at the URA3 chromosomal locus [YAB79::1A2(HIS3)] and the strain YAB79::1A2(2µ) expressing CYP1A2 from an episomal plasmid. The graphs represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

Although the CYP1A2 amounts were far more when expressed from the *HIS3* locus [i.e. from the cells in the strain YAB79::1A2(HIS3)] than from the *URA3* locus [i.e. from the cells in the strain YAB79::1A2(URA3)], yet the amounts produced by the latter strain were ~4-fold more than that produced by the strain [YAB79::1A2(2μ)] expressing CYP1A2 from the episomal, multi-copy plasmid pSYE263/CYP1A2_yc (Chapter 3, Figure 3.35). This would indicate that chromosomal integration provides a much more stable environment for expression of the CYP1A2 enzyme than when it is expressed from an episomal plasmid.

4.8 Construction of yeast strains, bearing expression cassettes of the human CYP2D6 variant genes, chemically synthesized using yeast biased codons, and comparison of CYP2D6 enzyme activities produced by different strains

4.8.1 Construction of a yeast integrative plasmid that would allow stable expression of human CYP2D6(1) enzyme from the URA3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpUraADH2S/CYP2D6(1)_yc [CYP2D6(1)_yc = gene coding for CYP2D6(1) (Val^{374}) protein (NCBI Accession Number, NM_000106) and synthesized using yeast-biased codons] was created via the following steps:

(1) After digestion of the vector YIpUraADH2S (Figure 4.15) with restriction enzymes *BamH*I, *Xba*I, a 5233 bp fragment was isolated. This eliminated 12 bp in the multi-cloning site.

(2) A 1524 bp *BamH*I-*Xba*I *CYP2D6(1)_yc* gene fragment was isolated from a pUC57 based plasmid into which the chemically synthesised gene had been cloned (obtained from Genewiz). *CYP2D6(1)_yc* gene was synthesized with yeast-biased codons using the human CYP2D6(1) protein as a template (Accession Number of protein sequence, NM_000106; Figure 4.40).

(3) The 5233 bp vector and the 1524 bp insert were ligated to obtain the plasmid YIpUraADH2S/CYP2D6(1)_yc (Figure 4.41).

```
  1  MGLEALVPLA VIVAIFLLLV DLMHRRQRWA ARYPPGPLPL PGLGNLLHVD FQNTPYCFDQ
 61  LRRRFGDVFS LQLAWTPVVV LNGLAAVREA LVTHGEDTAD RPPVPITQIL GFGPRSQGVF
121  LARYGPAWRE QRRFSVSTLR NLGLGKKSLE QWVTEEAACL CAAFANHSGR PFRPNGLLDK
181  AVSNVIASLT CGRRFEYDDP RFLRLLDLAQ EGLKEESGFL REVLNAVPVL LHIPALAGKV
241  LRFQKAFLTQ LDELLTEHRM TWDPAQPPRD LTEAFLAEME KAKGNPESSF NDENLRIVVA
301  DLFSAGMVTT STTLAWGLLL MILHPDVQRR VQQEIDDVIG QVRRPEMGDQ AHMPYTTAVI
361  HEVQRFGDIV PLGVTHMTSR DIEVQGFRIP KGTTLITNLS SVLKDEAVWE KPFRFHPEHF
421  LDAQGHFVKP EAFLPFSAGR RACLGEPLAR MELFLFFTSL LQHFSFSVPT GQPRPSHHGV
481  FAFLVSPSPY ELCAVPR
```

Figure 4.40. The protein sequence of CYP2D6(1), NM_000106, as published in the NCBI database (https://www.ncbi.nlm.nih.gov/). The residue Valine at position 374 is highlighted.

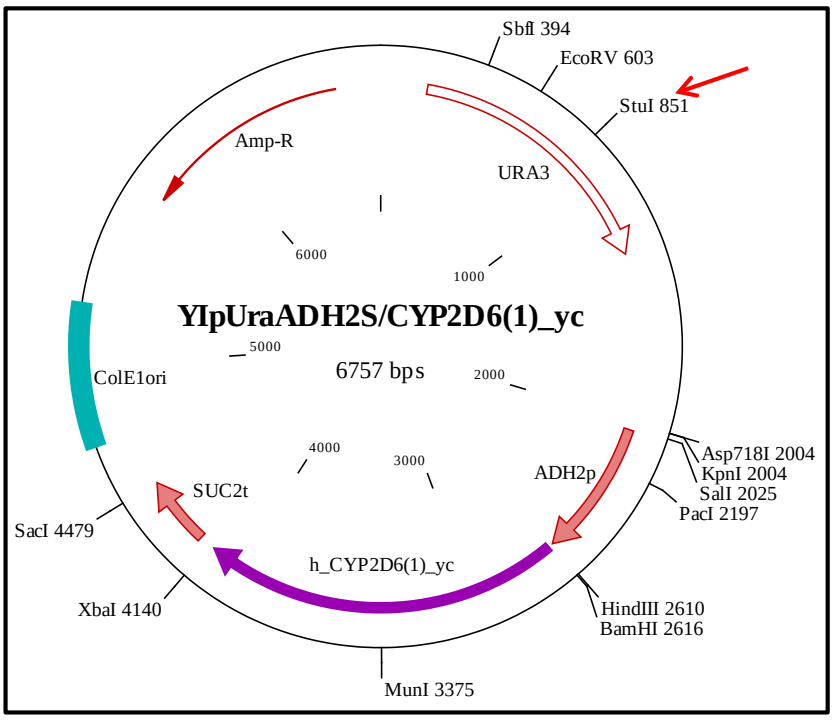


Figure 4.41. Map of plasmid YIpUraADH2S/CYP2D6(1)_yc that allows integration of a human CYP2D6(1) gene expression cassette at the URA3 locus of the yeast genome. The human h_CYP2D6(1) gene was synthesized using yeast-biased codons and was named CYP2D6(1)_yc. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpUraADH2S/CYP2D6(1)_yc was linearized at the *Stu*I site (as indicated by an arrow in Figure 4.41). Alternatively, the *EcoR*V site could have been used for linearization.

The newly constructed plasmid YIpUraADH2S/CYP2D6(1)_yc was further analysed via digestion with *Xho*I and *Bam*I + *Xho*I restriction enzymes (Figure 4.42). The agarose gels showed the expected sizes of DNA fragments.

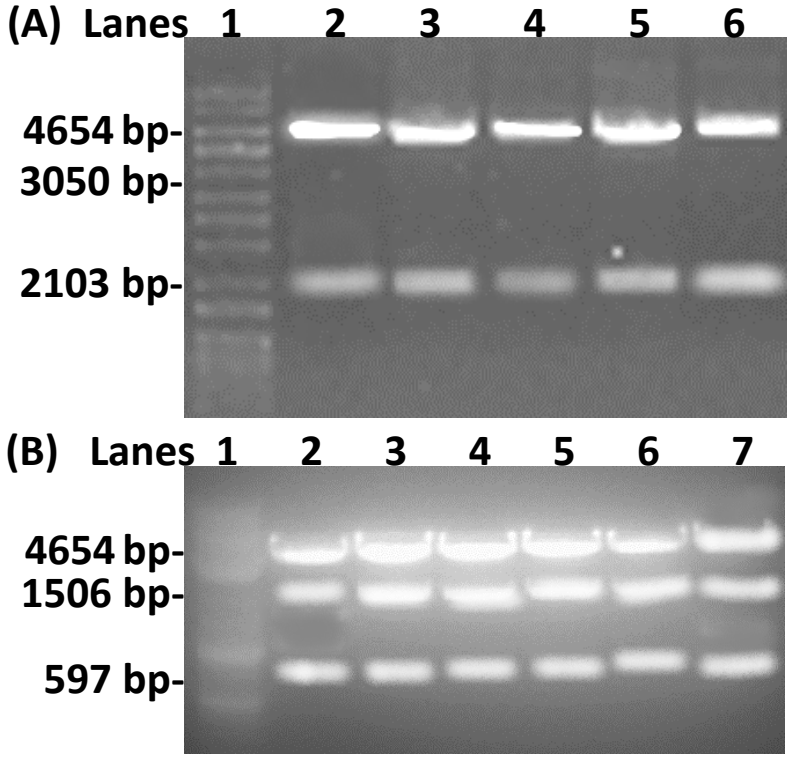


Figure 4.42. Agarose gels (A, B) that show the expected DNA fragments of YIpUraADH2S/CYP2D6(1)_yc when plasmids isolated from five (A) and six (B) bacterial clones were digested with XhoI (lanes 2-6; A) and BamHI-XhoI (lanes 2-7; B). Lane 1 (A & B), 2-log DNA ladder.

The YIpUraADH2S/CYP2D6(1)_yc plasmid can be used for expression of the *CYP2D6(1)_yc* gene driven by the *ADH2* promoter. In order to allow this, the *CYP2D6(1)_yc* gene expression cassette was integrated into the *URA3* locus on yeast chromosome V.

4.8.2 Construction of a yeast integrative plasmid that would allow stable expression of human CYP2D6(1) enzyme from the HIS3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpHisADH2S/CYP2D6(1)_yc was created via the following steps:

(1) After digestion of the vector YIpHisADH2S (Figure 4.11) with restriction enzymes *BamH*I, *Xba*I, a 5305 bp fragment was isolated. This eliminated 12 bp in the multi-cloning site.

(2) A 1524 bp *BamH*I-*Xba*I *CYP2D6(1)_yc* gene fragment (NCBI Accession Number, NM_000106) was isolated from a pUC57 based plasmid into which the chemically synthesised gene had been cloned (as in Section 4.6.1).

(3) The 5305 bp vector and the 1524 bp insert were ligated to obtain the plasmid YIpHisADH2S/ CYP2D6(1)_yc (Figure 4.43).

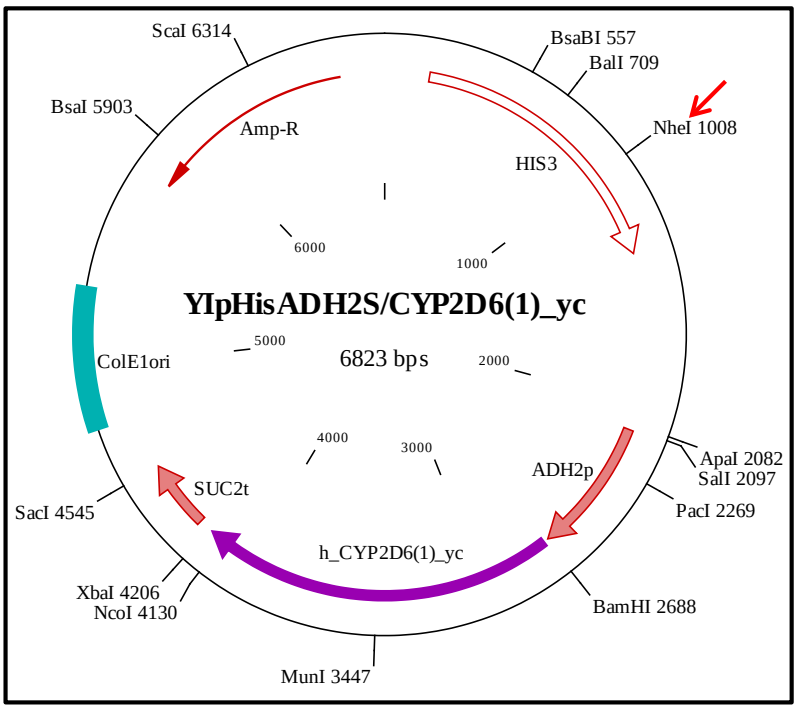


Figure 4.43. Map of plasmid YIpHisADH2S/CYP2D6(1)_yc that allows integration of a human CYP2D6(1) gene expression cassette at the HIS3 locus of the yeast genome. The human h_CYP2D6(1) gene was synthesized using yeast-biased codons and was named CYP2D6(1)_yc. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpHisADH2S/ CYP2D6(1)_yc was linearized at the *Nhe*I site (indicated by an arrow in Figure 4.43). In order to facilitate homologous recombination, the restriction sites *Bal*I or *BsaB*I (Figure 4.43) could also have been used for linearization.

The newly constructed plasmid YIpHisADH2S/CYP2D6(1)_yc was further analysed via digestion with *Xho*I and *Pvu*II restriction enzymes (Figure 4.44). The agarose gels showed the expected fragments.

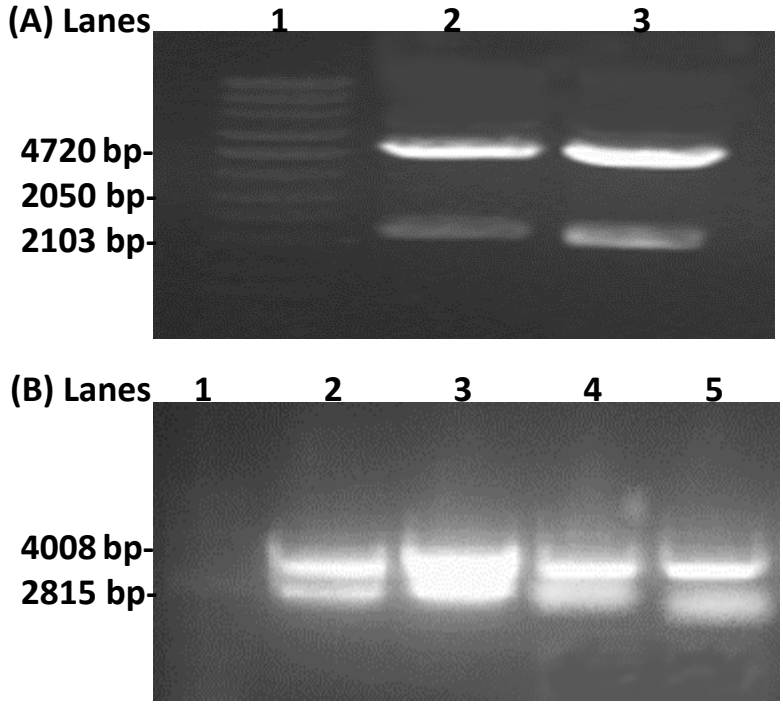


Figure 4.44. Agarose gels (A, B) that show the expected DNA fragments of YIpHisADH2S/CYP2D6(1)_yc when plasmids isolated from two (A) or four (B) bacterial clones were digested with XhoI (lanes 2-3; A) and PvuII (lanes 2-5; B). Lane 1 (A & B), 2-log DNA ladder [not visible in (B)].

The YIpHisADH2S/CYP2D6(1)_yc plasmid can be used for expression of the *CYP2D6(1)_yc* gene driven by the *ADH2* promoter. In order to allow this, the *CYP2D6(1)_yc* gene expression cassette would have to be integrated into the *HIS3* locus on chromosome XV.

4.8.3 Construction of yeast strains that contain a copy of the CYP2D6(1)_yc gene expression cassette integrated into two different chromosomal loci of the yeast strain YY7

Two yeast strains were generated that contained a single copy of the *CYPD6(1)_yc* gene expression cassette. They were obtained by individually integrating the plasmids that bear the *CYPD6(1)_yc* expression cassette in the yeast strain YY7 (BC300::ΔhRDM/LEU2$^+$)

that can express ΔhRDM from the *LEU2* locus. YY7 cells do not contain the human cytochrome *b5* gene.

The *CYPD6_yc* gene encoding plasmids used for integration in the strain YY7 were:

(1) YIpUraADH2S/CYP2D6(1)_yc, and

(2) YIpHisADH2S/CYP2D6(1)_yc.

After integration, the strains were named:

(a) YY7::2D6(1)_yc(URA3) and

(b) YY7::2D6(1)_yc(HIS3).

YAB79 (BC300::ΔhRDM/LEU2$^+$, b5/TRP1$^+$; Figure 26) has been explained in Section 4.4.4. It expresses ΔhRDM, a modified version of the human CYP450 reductase, hRD, the gene being expressed from the *ADH2* promoter. It also co-expresses human cytochrome b5 from the *GAPDH* promoter

The *CYPD6_yc* gene encoding plasmids used for integration in the strain YAB79 were:

After integration, the strains were named:

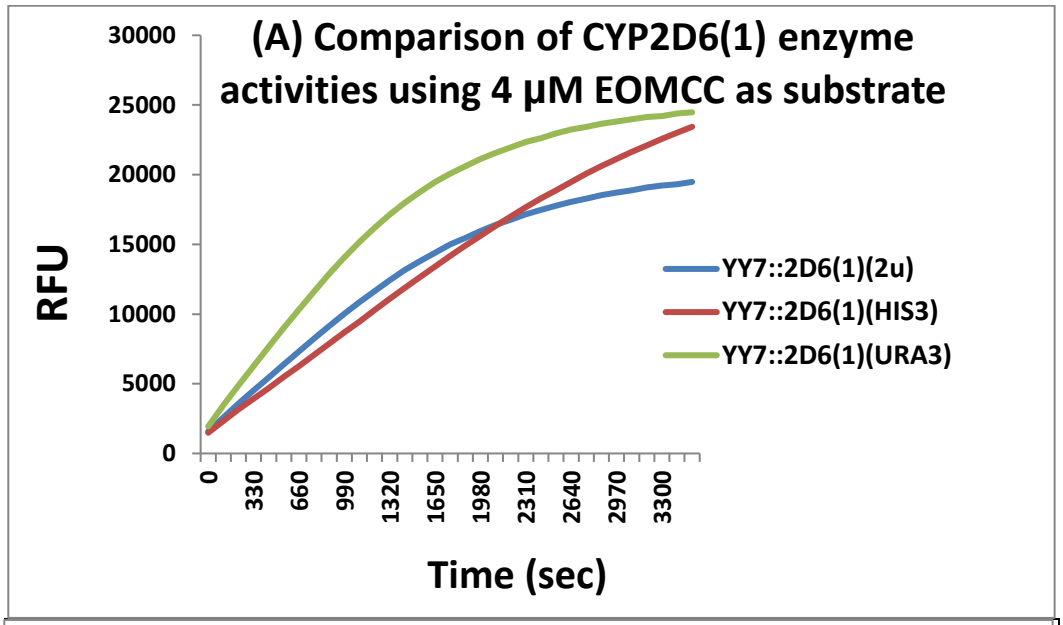
(a) YAB79::2D6(1)_yc(URA3) and

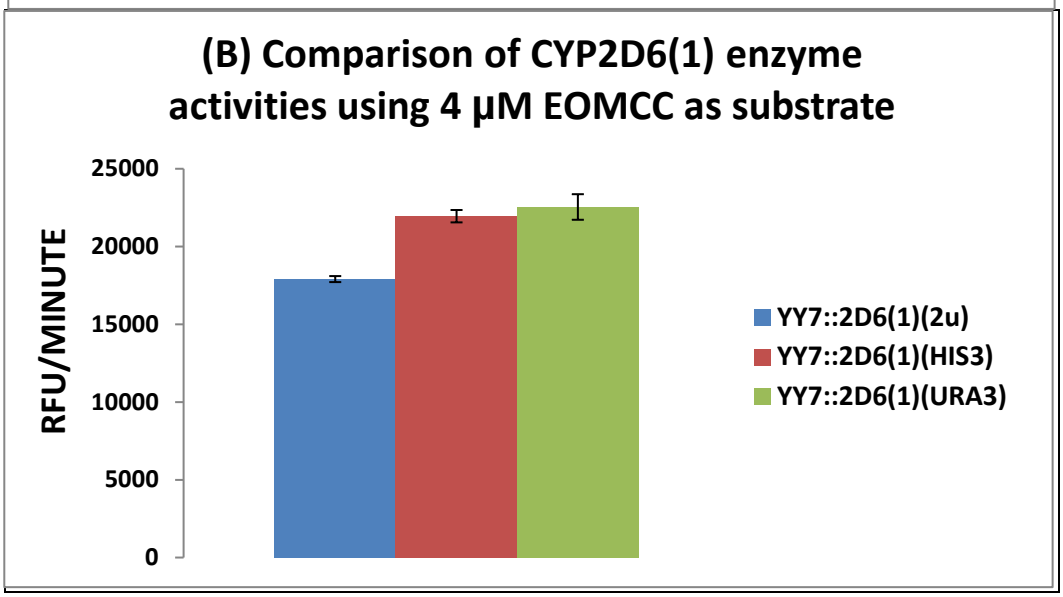
(b) YAB79::2D6 (1)_yc(HIS3).

The amounts of enzyme expressed from these strains were then compared in fluorescence assays using an appropriate fluorogenic substrate. Comparative kinetic analysis of CY2D6_yc co-expressed with and without cytochrome b5 in the presence of the modified

cytochrome P450 reductase (ΔhRDM), using EOMCC as a substrate, was performed (Figure 4.45). The intact yeast cells bearing the *CYP2D6(1)_yc* gene integrated in the chromosomal loci of *HIS3* and *URA3* genes, an episomal plasmid encoding the *CYP2D6(1)_yc* gene and the control empty plasmid contacting yeast strain were grown as described in Section 4.1.1 and the comparative enzyme kinetic assays were performed as described in Section 4.1.2. Formation of product, 7-HCC, was measured. These procedures were carried out in three independent experiments.

4.8.4 Comparison of activities of CYP2D6(1) enzyme expressed from (a) the CYP2D6(1)_yc gene chromosomally integrated at the HIS3 or URA3 locus, and (b) an episomal plasmid bearing the CYP2D6(1)_yc gene, in the yeast strains YY7 and YAB79





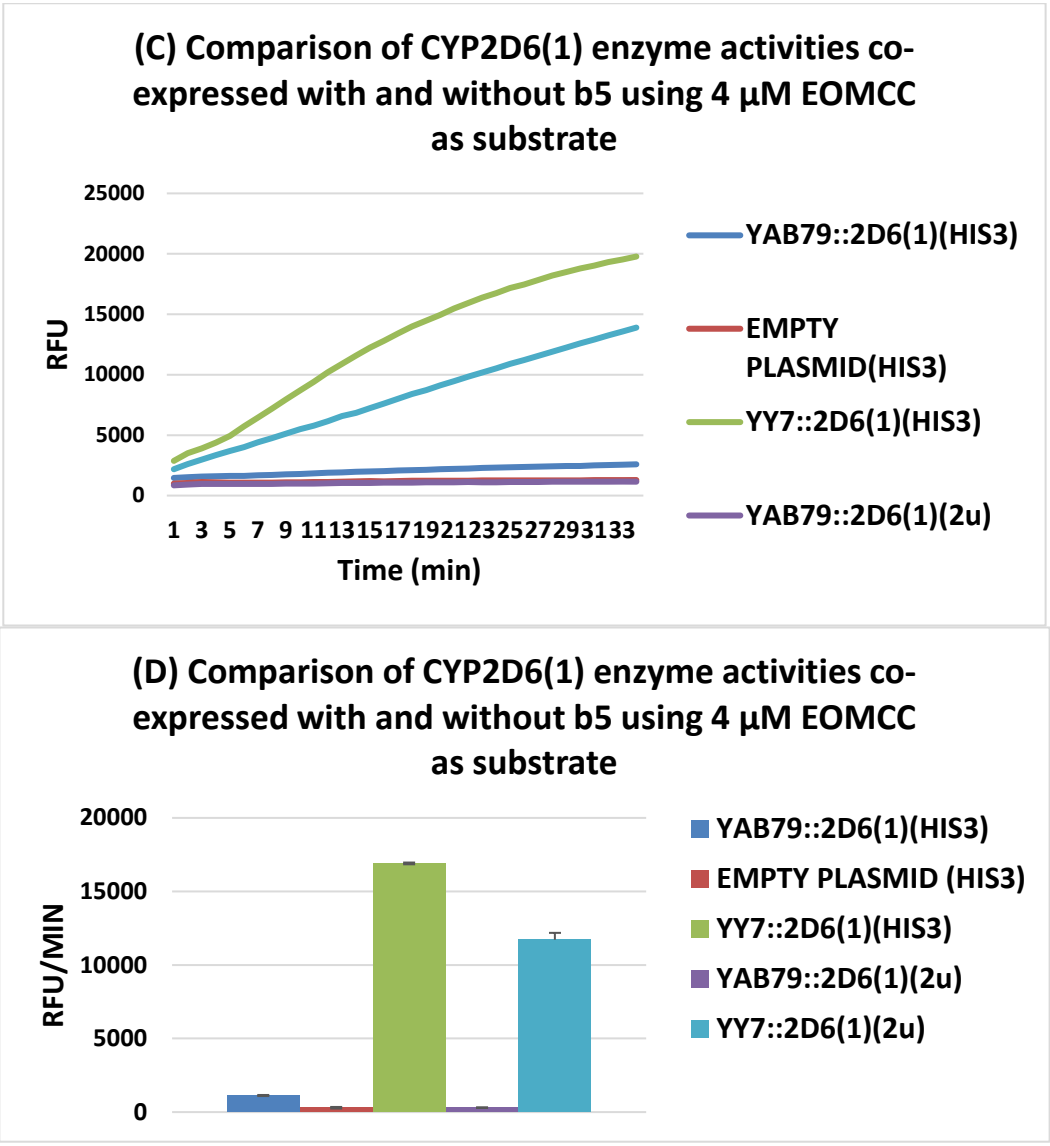


Figure 4.45. The graph (A) compares the CYP2D6(1) enzyme activities produced in the strains YY7::2D6(1)(HIS3) and YY7::2D6(1)(URA3), expressing a copy of the CYP2D6(1)_yc gene expression cassette, integrated at the HIS3 and URA3 chromosomal loci, and the strain YY7:: 2D6(1)(2µ) expressing CYPD6(1)_yc from an episomal, 2-micron (2u) plasmid. The graphs represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The graph (C) compares the CYP2D6(1) enzyme activities produced in the strains YAB79::2D6(1)(HIS3) and YY7::2D6(1)(HIS3), expressing a copy of the CYP2D6(1)_yc gene expression cassette, integrated at the HIS3 and URA3 chromosomal loci in the presence/absence of cytochrome b5, and similarly the strain YY7:: 2D6(1)(2µ) and YAB79::2D6(1)(2µ) expressing CYPD6(1)_yc from an episomal, 2-micron (2u) plasmid in the presence/absence of cytochrome b5. The graphs represent the average of results obtained from three independent experiments. The bar plot (D) mirrors the graphs in (C). The data represent mean ± S.D. of three independent experiments.

The graphs in Figure 45 (A) show the expression of a single copy of the *CYP2D6 (1)_yc* gene expression cassette from the *HIS3* locus is superior to that from the *URA3* locus. The CYP2D6 (1) enzyme expressed from the *HIS3* locus has a better activity than from the *URA3* locus implying that levels of CYP2D6 (1) expressed from the *HIS3* locus is more than that from the *URA3* locus. It should also be noted that both integrated copies produce higher amounts of CYP2D6 (1) enzyme than that from an episomal plasmid. Also the graphs in Figure 45 (C) show the expression of both the episomal plasmid (2µ) and single copy (*HIS3*) when co-expressed in the presence of cytochrome b5. The results show that b5 does not enhance the activity of CYP2D6 but, in contrary, tends to block the binding site of CYP2D6, making CYP2D6 activities collapse drastically compared to the enzyme expressed in the absence of cytochrome b5.

4.8.5 Construction of a yeast integrative plasmid that would allow stable expression of human CYP2D6(2) enzyme from the HIS3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpHisADH2S/CYP2D6(2)_yc was created via the following steps:

(1) The vector YIpHisADH2S (Figure 4.11) was digested with restriction enzymes *BamH*I, *Xba*I, and a 5305 bp fragment was isolated.

(2) A 1524 bp *BamH*I-*Xba*I *CYP2D6(2)_yc* gene fragment (coding for the Met^{374} wild-type variant) was isolated from a pUC57 based plasmid into which the chemically synthesised gene, using yeast-biased codons, had been cloned. The

gene was synthesized on the basis of the protein sequence published in the NCBI database (Accession Number, M20403; Figure 4.46).

(3) The 5305 bp vector and the 1524 bp insert were ligated to obtain the plasmid YIpHisADH2S/ CYP2D6(2)_yc (Figure 4.48).

```
  1  MGLEALVPLA VIVAIFLLLV DLMHRRQRWA ARYPPGPLPL PGLGNLLHVD FQNTPYCFDQ
 61  LRRRFGDVFS LQLAWTPVVV LNGLAAVREA LVTHGEDTAD RPPVPITQIL GFGPRSQGVF
121  LARYGPAWRE QRRFSVSTLR NLGLGKKSLE QWVTEEAACL CAAFANHSGR PFRPNGLLDK
181  AVSNVIASLT CGRRFEYDDP RFLRLLDLAQ EGLKEESGFL REVLNAVPVL LHIPALAGKV
241  LRFQKAFLTQ LDELLTEHRM TWDPAQPPRD LTEAFLAEME KAKGNPESSF NDENLRIVVA
301  DLFSAGMVTT STTLAWGLLL MILHPDVQRR VQQEIDDVIG QVRRPEMGDQ AHMPYTTAVI
361  HEVQRFGDIV PLGMTHMTSR DIEVQGFRIP KGTTLITNLS SVLKDEAVWE KPFRFHPEHF
421  LDAQGHFVKP EAFLPFSAGR RACLGEPLAR MELFLFFTSL LQHFSFSVPT GQPRPSHHGV
481  FAFLVSPSPY ELCAVPR
```

Figure 4.46. The protein sequence of CYP2D6(2), M20403, as published in the NCBI database (https://www.ncbi.nlm.nih.gov/).

```
                         10        20        30        40        50
                          *         *         *         *         *
2D6-1.PRT        MGLEALVPLAVIVAIFLLLVDLMHRRQRWAARYPPGPLPLPGLGNLLHVD
2D6-2.PRT        MGLEALVPLAVIVAIFLLLVDLMHRRQRWAARYPPGPLPLPGLGNLLHVD

                         60        70        80        90       100
                          *         *         *         *         *
2D6-1.PRT        FQNTPYCFDQLRRRFGDVFSLQLAWTPVVVLNGLAAVREALVTHGEDTAD
2D6-2.PRT        FQNTPYCFDQLRRRFGDVFSLQLAWTPVVVLNGLAAVREALVTHGEDTAD

                        110       120       130       140       150
                          *         *         *         *         *
2D6-1.PRT        RPPVPITQILGFGPRSQGVFLARYGPAWREQRRFSVSTLRNLGLGKKSLE
2D6-2.PRT        RPPVPITQILGFGPRSQGVFLARYGPAWREQRRFSVSTLRNLGLGKKSLE

                        160       170       180       190       200
                          *         *         *         *         *
2D6-1.PRT        QWVTEEAACLCAAFANHSGRPFRPNGLLDKAVSNVIASLTCGRRFEYDDP
2D6-2.PRT        QWVTEEAACLCAAFANHSGRPFRPNGLLDKAVSNVIASLTCGRRFEYDDP

                        210       220       230       240       250
                          *         *         *         *         *
2D6-1.PRT        RFLRLLDLAQEGLKEESGFLREVLNAVPVLLHIPALAGKVLRFQKAFLTQ
2D6-2.PRT        RFLRLLDLAQEGLKEESGFLREVLNAVPVLLHIPALAGKVLRFQKAFLTQ

                        260       270       280       290       300
                          *         *         *         *         *
2D6-1.PRT        LDELLTEHRMTWDPAQPPRDLTEAFLAEMEKAKGNPESSFNDENLRIVVA
2D6-2.PRT        LDELLTEHRMTWDPAQPPRDLTEAFLAEMEKAKGNPESSFNDENLRIVVA

                        310       320       330       340       350
                          *         *         *         *         *
2D6-1.PRT        DLFSAGMVTTSTTLAWGLLLMILHPDVQRRVQQEIDDVIGQVRRPEMGDQ
2D6-2.PRT        DLFSAGMVTTSTTLAWGLLLMILHPDVQRRVQQEIDDVIGQVRRPEMGDQ

                        360       370       380       390       400
                          *         *         *         *         *
2D6-1.PRT        AHMPYTTAVIHEVQRFGDIVPLGVTHMTSRDIEVQGFRIPKGTTLITNLS
2D6-2.PRT        AHMPYTTAVIHEVQRFGDIVPLGMTHMTSRDIEVQGFRIPKGTTLITNLS

                        410       420       430       440       450
                          *         *         *         *         *
2D6-1.PRT        SVLKDEAVWEKPFRFHPEHFLDAQGHFVKPEAFLPFSAGRRACLGEPLAR
2D6-2.PRT        SVLKDEAVWEKPFRFHPEHFLDAQGHFVKPEAFLPFSAGRRACLGEPLAR

                        460       470       480       490
                          *         *         *         *
2D6-1.PRT        MELFLFFTSLLQHFSFSVPTGQPRPSHHGVFAFLVSPSPYELCAVPR
2D6-2.PRT        MELFLFFTSLLQHFSFSVPTGQPRPSHHGVFAFLVSPSPYELCAVPR
```

Figure 4.47. Alignment of CYP2D6(1) and CYP2D6(2) protein sequences. The amino acid residue that differs (position 375) is highlighted.

The protein sequences of CYP2D6(1) and CYP2D6(2) differ in one amino acid residue at position 374 (Figure 4.47).

It has been suggested that the variant CYP2D6(1) (Val^{374}) produces much higher activity than CYP2D6(2) (Met^{374}) in insect cells (Yu et al., 2002). In fact, the leading manufacturer of recombinant human CYP enzymes (Corning-Gentest) distributes only CYP2D6(1) enzyme produced in insect cells.

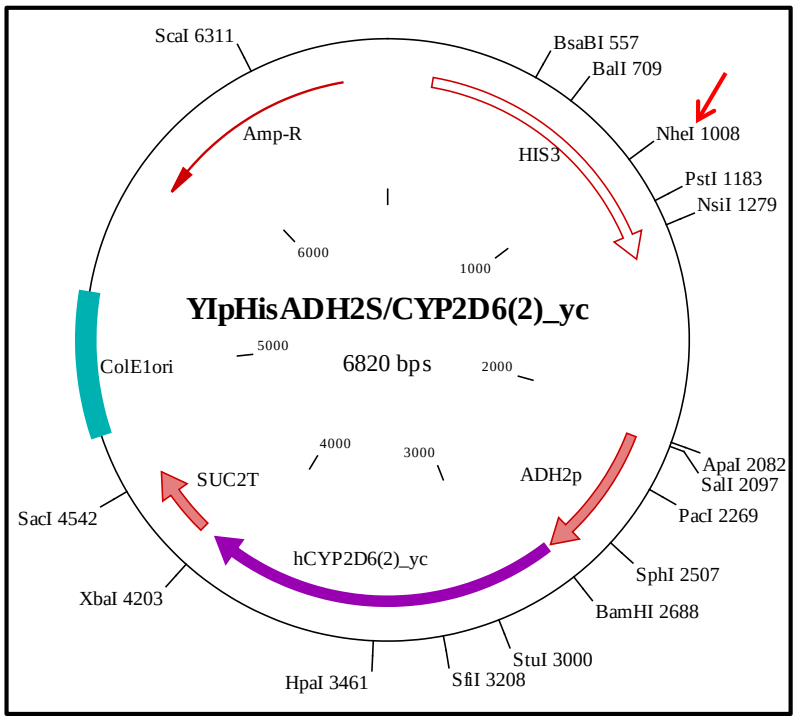


Figure 4.48. Map of plasmid YIpHisADH2S/CYP2D6(2)_yc that allows integration of a human *CYP2D6*(2) gene expression cassette at the *HIS3* locus of the yeast genome. The human *CYP2D6*(2) gene was synthesized using yeast-biased codons and was named hCYP2D6(2)_yc. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpHisADH2S/ CYP2D6(2)_yc was linearized at the *Nhe*I site (indicated by an arrow in

Figure 4.48). In order to facilitate homologous recombination, the restriction sites *Bal*I or *BsaB*I (Figure 4.48) could also have been used for linearization.

The newly constructed plasmid YIpHisADH2S/CYP2D6(2)_yc was further analysed via digestion with *Xho*I and *BamH*I-*Xba*I restriction enzymes (Figure 4.49). The agarose gels show the expected fragments.

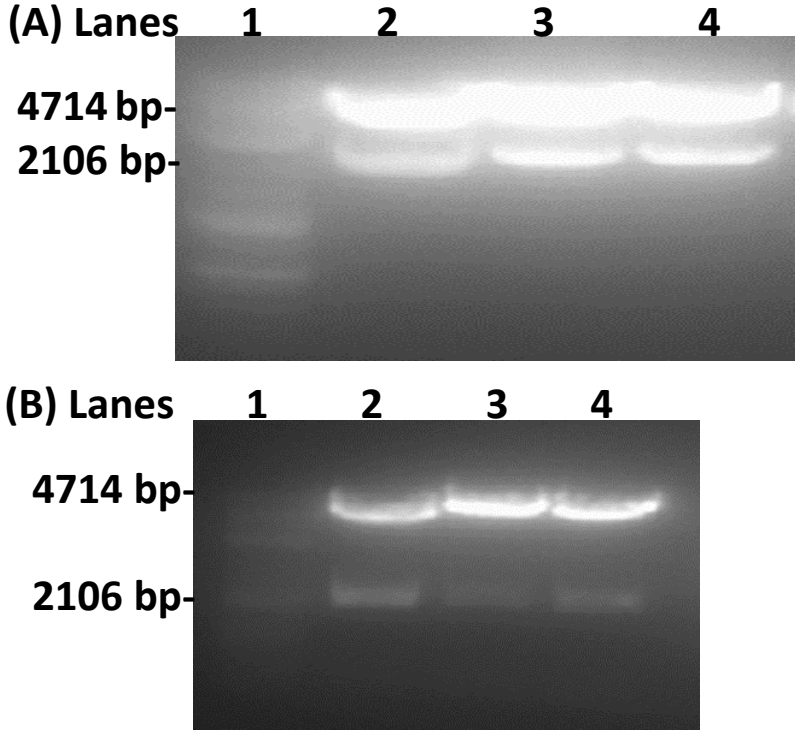


Figure 4.49. Agarose gels (A, B) that show the expected DNA fragments of YIpHisADH2S/CYP2D6(2)_yc when plasmids isolated from three bacterial clones (A and B) were digested with XhoI (lanes 2-4; A & B) and BamHI-XbaI (lanes 2-4; A & B). Lane 1 (A & B), 2-log DNA ladder [not clearly visible in (B)].

The plasmid YIpHisADH2S/CYPD6(2)_yc can be used for expression of the *CYP2D6(2)_yc* gene driven by the *ADH2* promoter. In order to allow this, the *CYP2D6(2)_yc* gene expression cassette must be integrated into the *HIS3* locus on chromosome XV of the strain YY7.

4.8.6 Construction of a yeast integrative plasmid that would allow stable expression of human CYP2D6(2) enzyme from the URA3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpUraADH2S/CYP2D6(2)_yc was created via the following steps:

(1) The vector YIpUraADH2S (Figure 4.15) was digested with restriction enzymes *BamH*I, *Xba*I, and a 5233 bp fragment was isolated.

(2) A 1524 bp *BamH*I-*Xba*I *CYP2D6(2)_yc* gene fragment was isolated from the pUC57 based plasmid, as in Section 4.6.5.

(3) The 5233 bp vector and the 1524 bp insert were ligated to obtain the plasmid YIpUraADH2S/ CYP2D6(2)_yc (Figure 4.50).

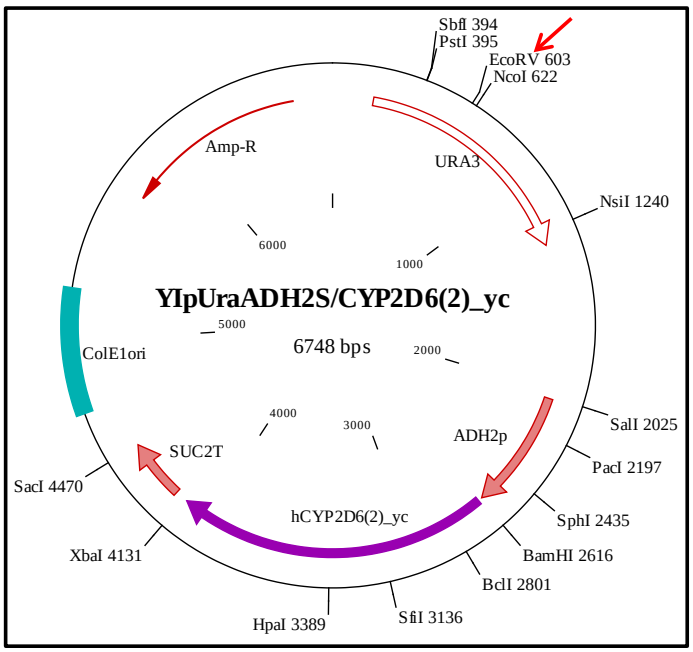


Figure 4.50. Map of plasmid YIpUraADH2S/CYP2D6(2)_yc that allows integration of a human *CYP2D6*(2) gene expression cassette at the *URA3* locus of the yeast genome. The human *CYP2D6*(2) gene was synthesized using yeast-biased codons and was named hCYP2D6(2)_yc. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpUraADH2S/CYP2D6(2)_yc was linearized at the *Eco*RV site (as indicated by an arrow in Figure 4.40). Alternatively, the *Nco*I site could have been used for linearization.

The newly constructed plasmid YIpUraADH2S/CYP2D6(2)_yc was further analysed via digestion with *Bam*I + *Xba*I restriction enzymes (Figure 4.51). The agarose gel showed the expected sizes of DNA fragments.

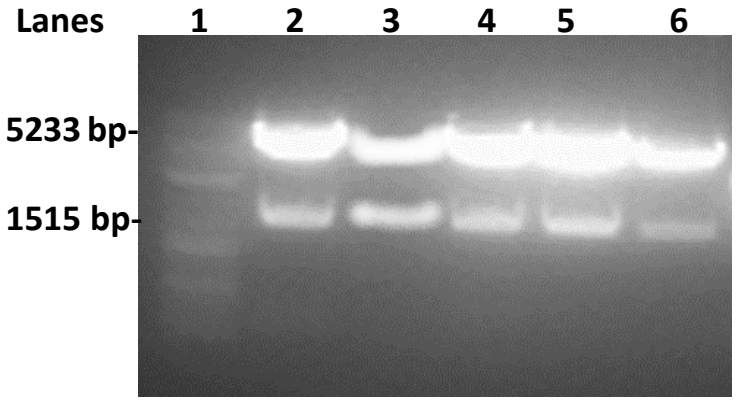


Figure 4.51. Agarose gel that shows the expected DNA fragments of YIpUraADH2S/CYP2D6(2)_yc when plasmids isolated from five bacterial clones were digested with BamHI-XbaI (lanes 2-6). Lane 1, 2-log DNA ladder.

The YIpUraADH2S/CYPD6(2)_yc plasmid was used for expression of the *CYP2D6(2)_yc* gene driven by the *ADH2* promoter. In order to allow this, the *CYP2D6(2)_yc* gene expression cassette was integrated into the *URA3* locus on chromosome V.

4.8.7 Construction of yeast strains that contain a copy of the CYP2D6(2)_yc gene expression cassette integrated into two different chromosomal loci of the yeast strain YY7 and YAB79

Two yeast strains were generated that contained a single copy of the *CYP2D6(2)_yc* gene expression cassette. They were obtained by individually integrating the plasmids that bear the *CYP2D6(2)_yc* expression cassette in the yeast strain YY7 (BC300::ΔhRDM/LEU2$^+$) that can express ΔhRDM from the *LEU2* locus. YY7 cells do not contain the human cytochrome *b5* gene.

The *CYP2D6(2)_yc* gene encoding plasmids used for integration in the strain YY7 were:

(1) YIpUraADH2S/CYP2D6(2)_yc, and

(2) YIpHisADH2S/CYP2D6(2)_yc.

After integration, the strains were named:

(a) YY7::2D6(2)_yc(URA3) and

(b) YY7::2D6(2)_yc(HIS3).

The amounts of enzyme expressed from these strains were then compared in fluorescence assays using an appropriate fluorogenic substrate. The comparative kinectic analysis of CY2D6(2)_yc co-expressed without cytochrome b5 with cytochrome P450 reductase (CPR) using EOMCC as a substrate. The intact yeast cells bearing the CYP2D6(2) integrated in chromosomal loci (HIS3 and URA3), the episomal CYP2D6(2)_yc and the control empty plasmid yeast strain were grown as described in section 4.1.1 and the comparative enzyme kinetic assays were measure as described in section 4.1.2 to measure product, 7-HCC formation. These procedures were carried out in three different experiments.

YAB79 (BC300::ΔhRDM/LEU2$^+$, b5/TRP1$^+$; Figure 26) has been explained in Section 4.4.4. It expresses ΔhRDM, a modified version of the human CYP450 reductase, hRD, the gene being expressed from the *ADH2* promoter. It also co-expresses human cytochrome b5 from the *GAPDH* promoter

The *CYPD6(2)_yc* gene encoding plasmids used for integration in the strain YAB79 were:

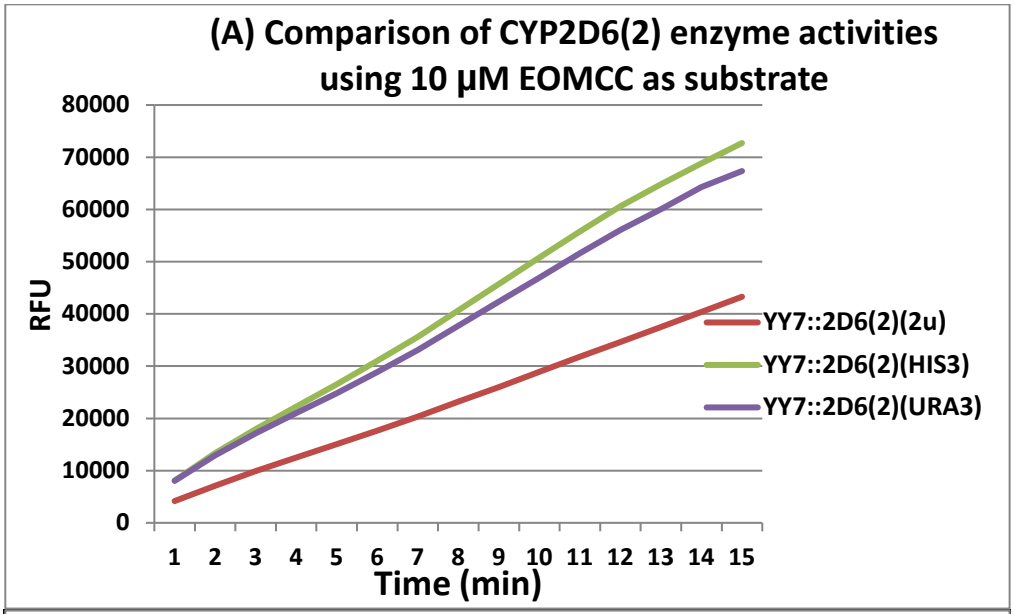
After integration, the strains were named:

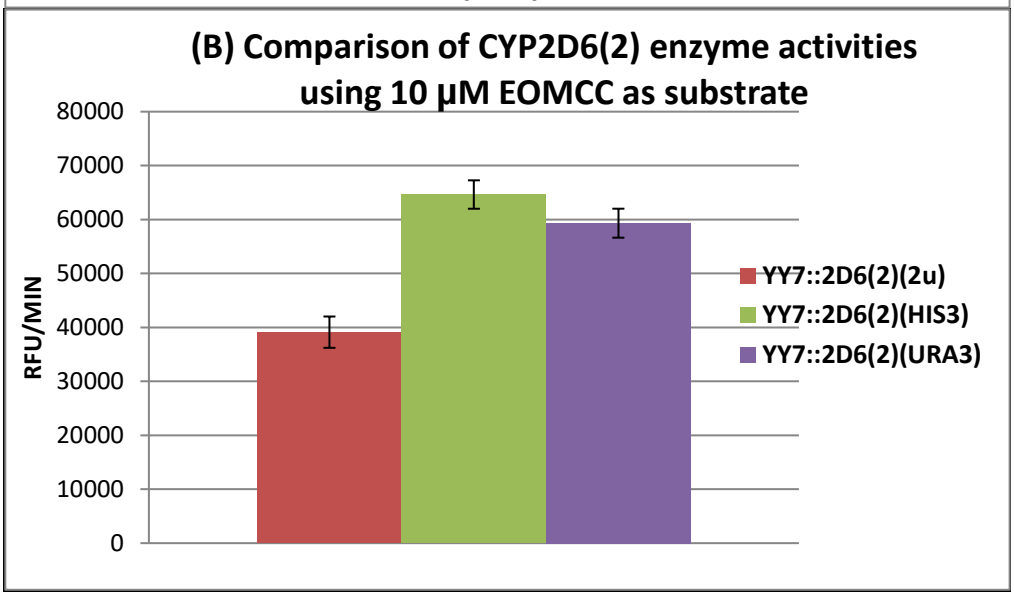
(a) YAB79::2D6(2)_yc(URA3) and

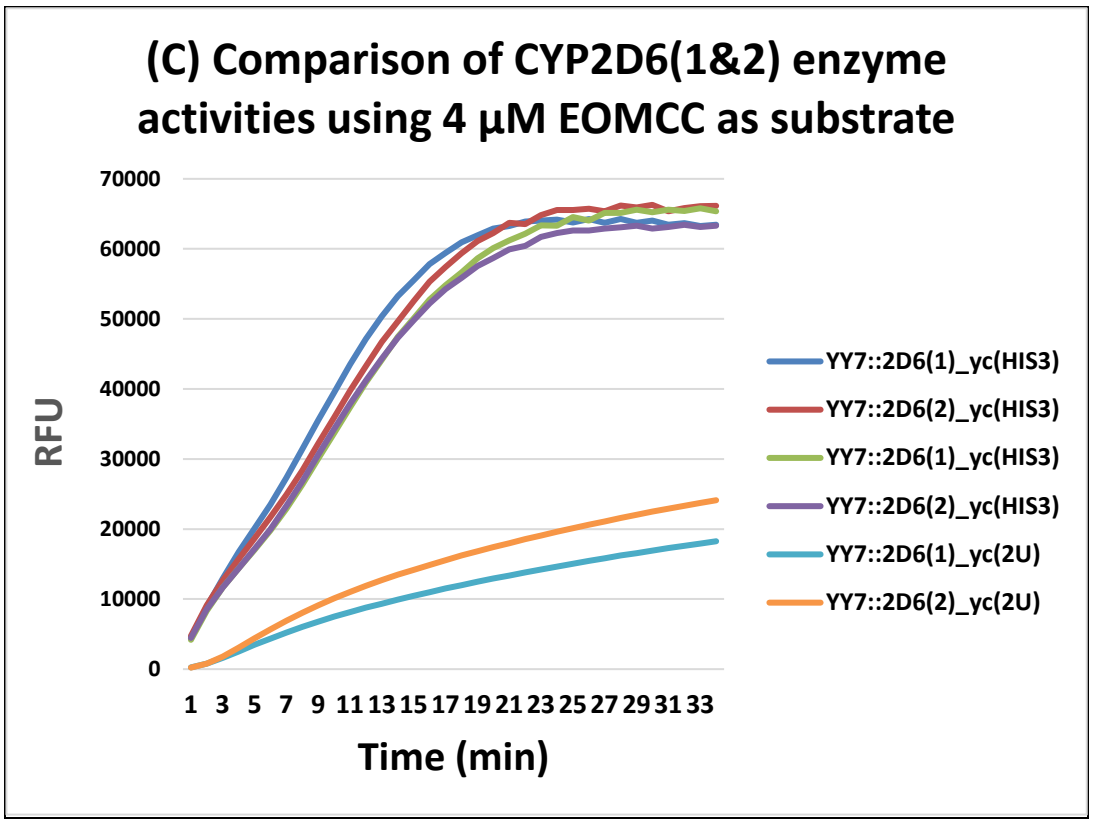
(b) YAB79::2D6 (2)_yc(HIS3).

The amounts of enzyme expressed from these strains were then compared in fluorescence assays using an appropriate fluorogenic substrate. Comparative kinetic analysis of CY2D6(2)_yc co-expressed with and without cytochrome b5 in the presence of the modified cytochrome P450 reductase (ΔhRDM), using EOMCC as a substrate, was performed (Figure 4.45). The intact yeast cells bearing the *CYP2D6(2)_yc* gene integrated in the chromosomal loci of *HIS3* and *URA3* genes, an episomal plasmid encoding the *CYP2D6(2)_yc* gene and the control empty plasmid contacting yeast strain were grown as described in Section 4.1.1 and the comparative enzyme kinetic assays were performed as described in Section 4.1.2. Formation of product, 7-HCC, was measured. These procedures were carried out in three independent experiments.

4.8.8 Comparison of activities of CYP2D6(2) enzyme expressed from (a) the CYP2D6(2)_yc gene chromosomally integrated at the HIS3 or URA3 locus, and (b) an episomal plasmid bearing the CYP2D6(2)_yc gene, in the yeast strain YY7; comparison of CYP2D6(1) and CYP2D6(2) enzyme activities






(C) Comparison of CYP2D6(1&2) enzyme activities using 4 µM EOMCC as substrate
RFU
70000
60000
50000
40000
30000
20000
10000
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33
Time (min)
YY7::2D6(1)_yc(HIS3)
YY7::2D6(2)_yc(HIS3)
YY7::2D6(1)_yc(HIS3)
YY7::2D6(2)_yc(HIS3)
YY7::2D6(1)_yc(2U)
YY7::2D6(2)_yc(2U)

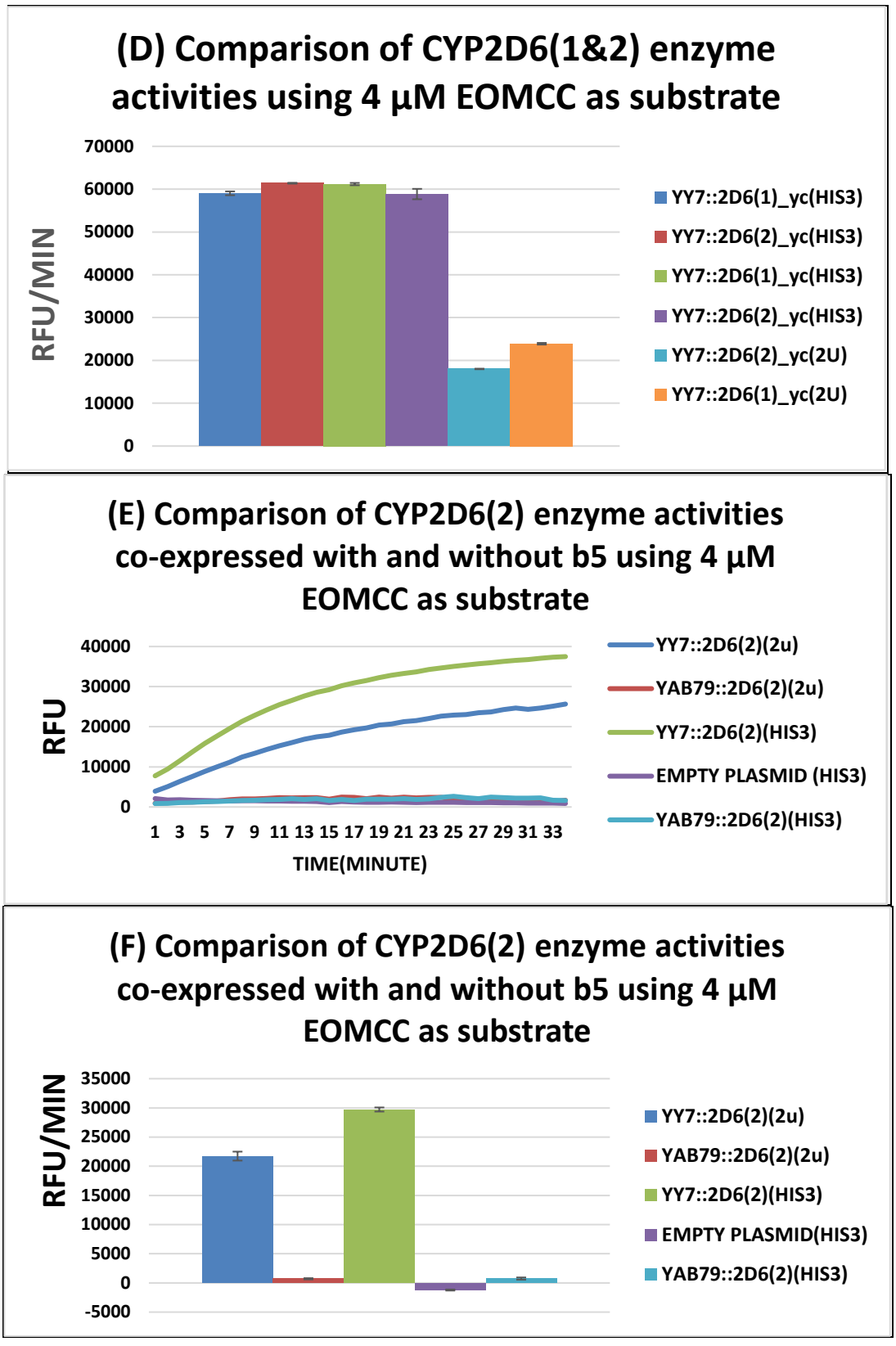


Figure 4.52. The graph (A) compares the CYP2D6(2) enzyme activities produced in the strains YY7::2D6(2)(HIS3) and YY7::2D6(2)(URA3), expressing a copy of the CYP2D6(2)_yc gene expression cassette, integrated at

the HIS3 and URA3 chromosomal loci, and the strain YY7::2D6(2)(2µ) expressing CYPD6(2)_yc from an episomal, 2-micron (2u) plasmid. The graphs represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments. The graph (C) compares the activities of the CYP2D6(1) and CYP2D6(2) enzymes. The bar plot (D) represents units emitted at 30 min of reaction with the substrate, EOMCC. The graph (E) compares the CYP2D6(2) enzyme activities produced in the strains YAB79::2D6(2)(HIS3) and YY7::2D6(2)(HIS3), expressing a copy of the CYP2D6(2)_yc gene expression cassette, integrated at the HIS3 chromosomal loci in the presence/absence of cytochrome b5, and similarly the strain YY7:: 2D6(2)(2µ) and YAB79::2D6(2)(2µ) expressing CYPD6(2)_yc from an episomal, 2-micron (2u) plasmid in the presence/absence of cytochrome b5. The graphs represent the average of results obtained from three independent experiments. The bar plot (F) mirrors the graphs in (E). The data represent mean ± S.D. of three independent experiments.

Figure 4.52 shows that the expression of a single copy of the *CYP2D6(2)_yc* gene from the *HIS3* locus is superior to that from the *URA3* locus. The CYP2D6(2) enzyme expressed from the *HIS3* locus has a better activity than from the *URA3* locus which may reflect that the enzyme produced from the *HIS3* locus is more than that produced from the *URA3* locus. However, once again, both integrated copies of the gene produce higher amounts than that obtained from an episomal plasmid.

Also the graphs in Figure 4.52 (E) show the expression of both the episomal plasmid (2µ) and single copy (*HIS3*) when co-expressed in the presence of cytochrome b5. The results show that b5 does not enhance the activity of CYP2D6(2) but, in contrary, tends to block the binding site of CYP2D6(2), making CYP2D6(2) activities collapse drastically compared to the enzyme expressed in the absence of cytochrome b5.

4.9 Construction of yeast strains, bearing expression cassettes of the human CYP2C19 gene, chemically synthesized using yeast biased codons, and comparison of CYP2C19 enzyme activities produced by different strains

4.9.1 Construction of a yeast integrative plasmid that would allow stable expression of human CYP2C19 enzyme from the HIS3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpHisADH2S/CYP2C19_yc was created via the following steps:

(1) After digestion of the plasmid YIpHisADH2S (Figure 4.11) with restriction enzymes *BamH*I, *Xba*I, the 5305 bp vector fragment was isolated.

(2) A 1485 bp *BamH*I-*Xba*I *CYP2C19_yc* gene fragment was isolated from a pUC57 plasmid. The gene was synthesized (Genewiz), using yeast-biased codons, based on the sequence of the human CYP2C19 protein with NCBI Accession Number, NP_000760. The gene was isolated from the pUC57 based plasmid into which the chemically synthesised gene had been cloned.

(3) The 5305 bp vector and the 1485 bp insert were ligated to create the plasmid YIpHisADH2S/CYP2C19_yc (Figure 4.53).

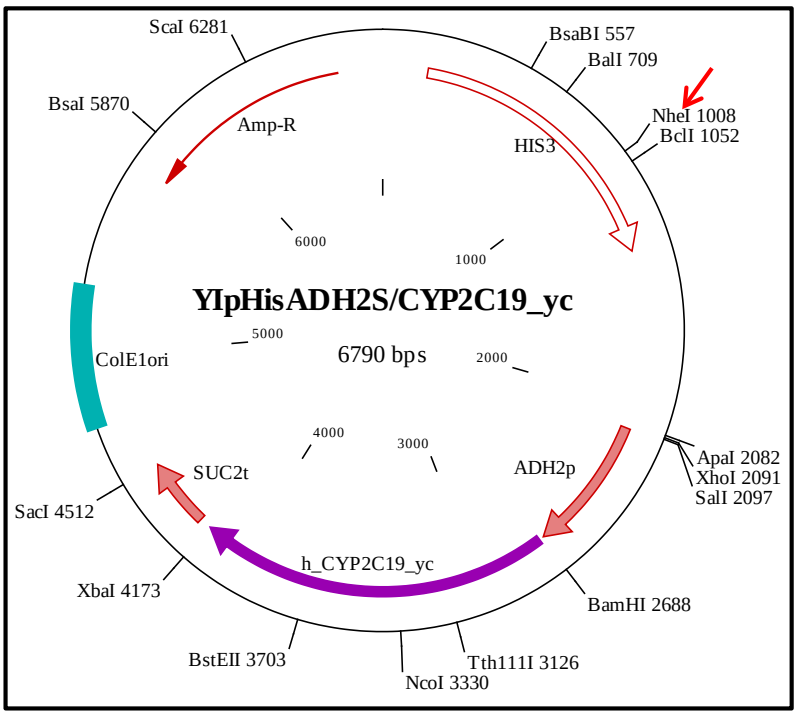


Figure 4.53. Map of plasmid YIpHisADH2S/CYP2C19_yc that allows integration of a human CYP2C19 gene expression cassette at the HIS3 locus of the yeast genome. The human CYP2C19 gene was synthesized using yeast-biased codons and was named h_CYP2C19_yc. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpHisADH2S/CYP2C19_yc was linearized at the *Nhe*I site (indicated by the arrow in Figure 4.53). In order to facilitate homologous recombination, the restriction sites *Bal*I, *BsaB*I or *Bcl*I (Figure 4.53) could also have been used for linearization.

The newly constructed plasmid YIpHisADH2S/CYP2C19_yc was further analysed via digestion with *BamH*I-*Xba*I (double-digest) and *Pst*I restriction enzymes (Figure 4.54). The agarose gels show expected sizes of the DNA fragments.

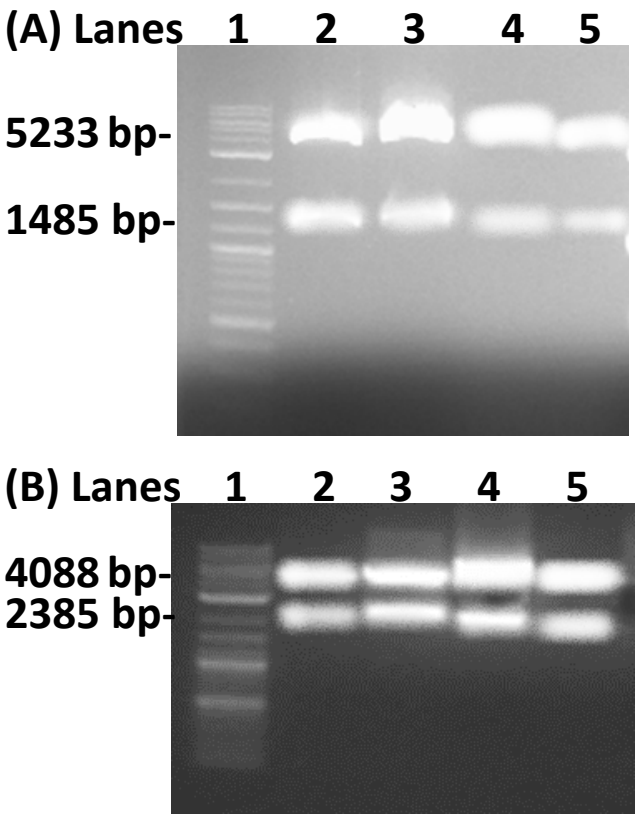


Figure 4.54. Agarose gels (A, B) that show the expected DNA fragments of YIpHisADH2S/CYP2C19_yc when plasmids isolated from four bacterial clones were digested with BamHI-XbaI (lanes 2-5) and PstI (lanes 2-5). Lane 1 (A & B), 2-log DNA ladder.

The YIpHisADH2S/CYP2C19_yc plasmid can be used for expression of the *CYP2C19_yc* gene driven by the *ADH2* promoter. In order to allow this, the *CYP2C19_yc* gene expression cassette was integrated into the *HIS3* locus on chromosome XV.

4.9.2 Construction of a yeast integrative plasmid that would allow stable expression of human CYP2C19 enzyme from the URA3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpUraADH2S/CYP2C19_yc [CYP2C19_yc = gene coding for CYP2C19 protein (as in Section 4.7.1; Accession Number NP_000760) and synthesized using yeast-biased codons] was created via the following steps:

(1) After digestion of the vector YIpUraADH2S (Figure 4.15) with restriction enzymes *BamH*I, *Xba*I, a 5233 bp fragment was isolated. This eliminated 12 bp in the multi-cloning site.

(2) A 1485 bp *BamH*I-*Xba*I *CYP2C19_yc* gene fragment was isolated from a pUC57 based plasmid (as in Section 4.7.1).

(3) The 5233 bp vector and the 1485 bp insert were ligated to obtain the plasmid YIpUraADH2S/CYP2C19_yc (Figure 4.55).

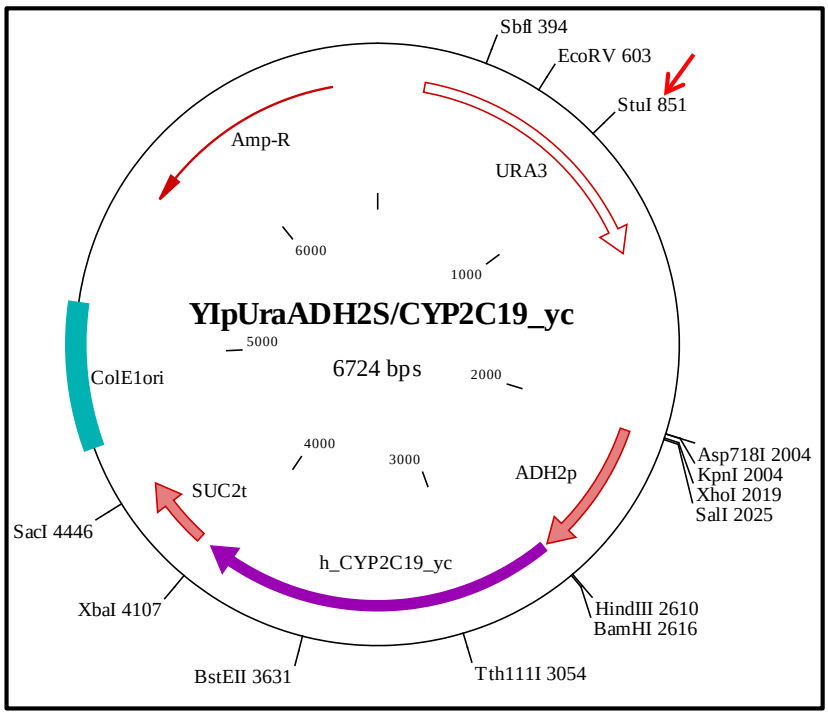


Figure 4.55. Map of plasmid YIpUraADH2S/CYP2C19_yc that allows integration of a human CYP2C19 gene expression cassette at the URA3 locus of the yeast genome. The human CYP2C19 gene was synthesized using yeast-biased codons and was named h_CYP2C19. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpUraADH2S/CYP2C19_yc was linearized at the *Stu*I site (as indicated by the arrow in Figure 4.55). Alternatively, the *Eco*RV site could also have been used for linearization.

The newly constructed plasmid YIpUraADH2S/ CYP2C19 was further analysed via digestion with *BamH*I-*Xba*I (double-digest) and *Nco*I restriction enzymes (Figure 4.56). The agarose gels show the expected sizes of DNA fragments.

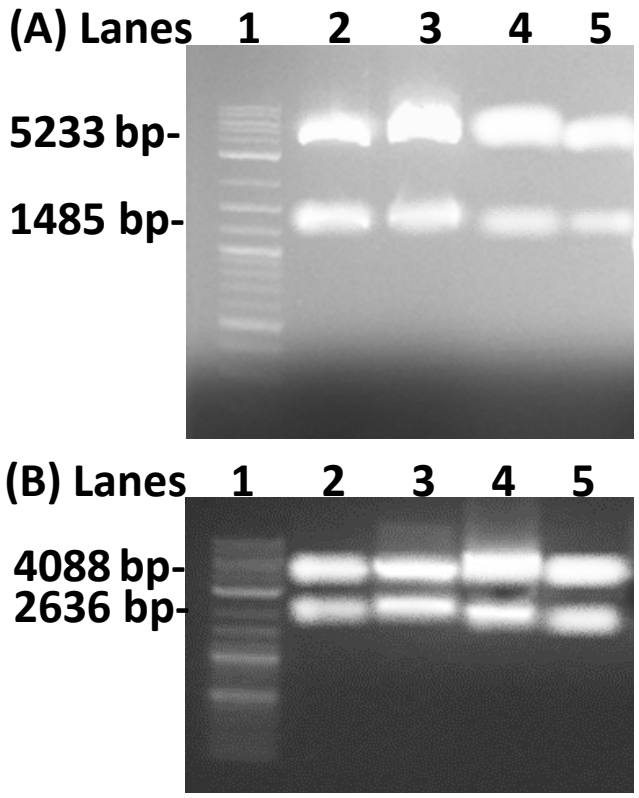


Figure 4.56. Agarose gels (A, B) that show the expected DNA fragments of YIpUraADH2S/CYP2C19_yc when plasmids isolated from four bacterial clones were digested with BamHI-XbaI (lanes 2-5) and NcoI (lanes 2-5). Lane 1 (A & B), 2-log DNA ladder.

The plasmid YIpUraADH2S/CYP2C19_yc can be used for expression of the *CYP2C19_yc* gene driven by the *ADH2* promoter. In order to allow this, the *CYP2C19_yc* gene expression cassette must be integrated at the *URA3* locus on chromosome V of yeast cells.

4.9.3 Construction of yeast strains that contain a copy of the CYP2C19_yc gene expression cassette integrated into two different chromosomal loci of the yeast strain YAB79

Two yeast strains were generated that contained a single copy of the *CYP2C19_yc* gene expression cassette. They were obtained by individually integrating the plasmids that bear the *CYP2C19_yc* expression cassette in the yeast strain YAB79 (BC300::ΔhRDM/LEU2$^+$, b5/TRP1$^+$; Figure 4.26) that express ΔhRDM from the *LEU2* locus and cytochrome b5 from the *TRP1* locus.

The *CYP2C19_yc* gene encoding plasmids used for integration in the strain YAB79 were:

(1) YIpUraADH2S/CYP2C19_yc, and

(2) YIpHisADH2S/ CYP2C19_yc.

After integration, the strains were named:

(a) YAB79::2C19_yc(URA3) and

(b) YAB79::2C19_yc(HIS3).

The amounts of enzyme expressed from these strains were then compared in fluorescence assays using an appropriate fluorogenic substrate. The kinetic analysis of CY2C19 enzyme co-expressed with cytochrome b5 and cytochrome P450 reductase ΔhRDM, using CEC as a substrate, is shown in Figure 4.57. The intact yeast cells bearing the *CYP2C19_yc* gene integrated in chromosomal loci *HIS3* and *URA3*, the episomal plasmid encoding the *CYP2C19_yc* gene and the control empty plasmid containing yeast strain were grown as described in section 4.1.1 and the comparative enzyme kinetic assays were

measure as described in section 4.1.2 to measure product, CHC formation. These procedures were carried out in three independent experiments.

4.9.4 Comparison of activities of CYP2C19 enzyme expressed from (a) the CYP2C19_yc gene chromosomally integrated at the HIS3 or URA3 locus, and (b) an episomal plasmid bearing the CYP2C19_yc gene, in the yeast strain YAB79

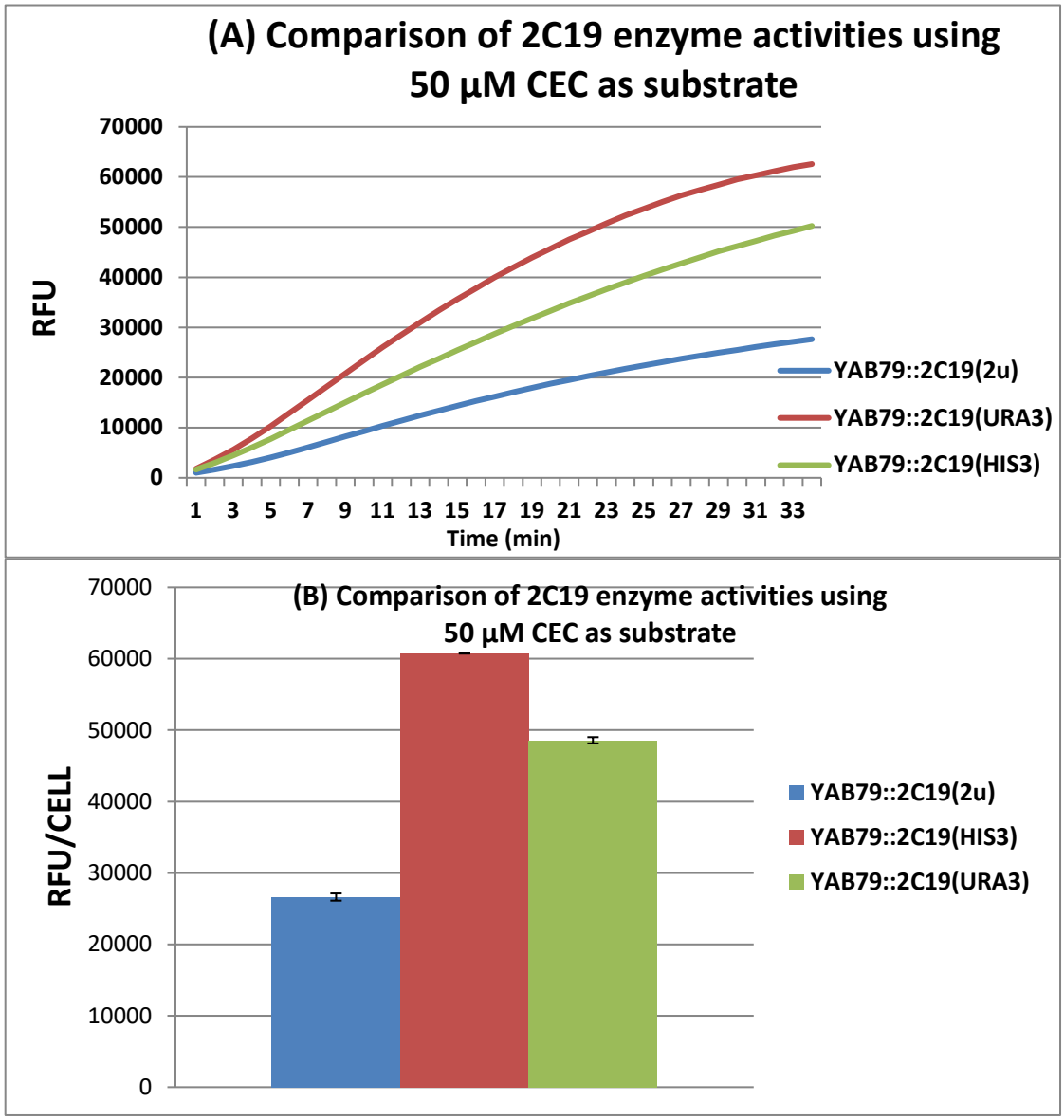


Figure 4.57. The graph (A) compares the CYP2C19 enzyme activities produced in the strains YAB79::2C19(HIS3) and YAB79::2C19(URA3), expressing a copy of the CYP2C19_yc gene expression cassette, integrated at the HIS3 and URA3 chromosomal loci, and the strain YAB79::2C19(2µ) expressing CYP2C19_yc from an episomal, 2-micron (2u) plasmid. The graphs represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

Figure 4.57 shows that the expression of a single copy of the *CYPC19_yc* gene from the *HIS3* locus is superior to that from the *URA3* locus. The CYP2C19 enzyme expressed from the *HIS3* locus has a better activity than from the *URA3* locus which may reflect that the enzyme produced from the *HIS3* locus is more than that produced from the *URA3* locus. However, once again, both integrated copies of the gene produce higher amounts than that obtained from an episomal plasmid.

4.10 Construction of yeast strains, bearing expression cassettes of the human CYP2C9 gene, chemically synthesized using yeast biased codons, and comparison of CYP2C9 enzyme activities produced by different strains

4.10.1 Construction of a yeast integrative plasmid that would allow stable expression of human CYP2C9 enzyme from the URA3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpUraADH2S/CYP2C9_yc [CYP2C9_yc = gene coding for CYP2C9 protein (Accession Number AAI25055) and synthesized using yeast-biased codons] was created via the following steps:

(1) The plasmid YIpUraADH2S (Figure 4.15) digested with the restriction enzymes *BamH*I, *Xba*I, and a 5233 bp vector fragment was isolated.

(2) A 1485 bp *BamH*I-*Xba*I *CYP2C9_yc* gene fragment was isolated from a pUC57 based plasmid into which the human *CYP2C9* gene, chemically synthesized using yeast-biased codons (Genewiz), was cloned.

(3) The 5233 bp vector and the 1485 bp insert were ligated to obtain the plasmid YIpUraADH2S/CYP2C9_yc (Figure 4.58).

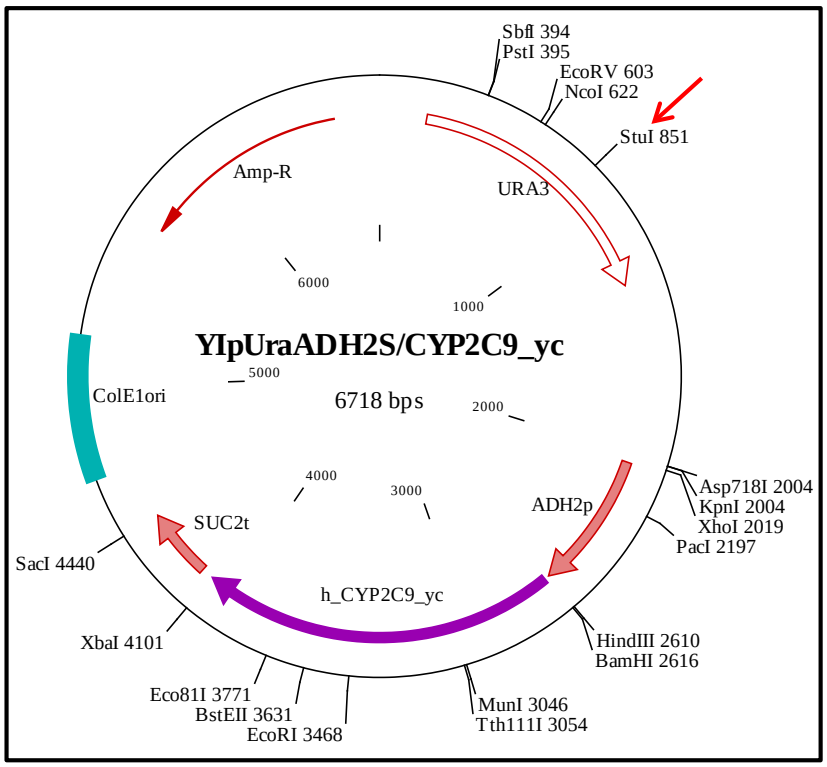


Figure 4.58. Map of plasmid YIpUraADH2S/CYP2C9_yc that allows integration of a human CYP2C9 gene expression cassette at the URA3 locus of the yeast genome. The human CYP2C9 gene was synthesized using yeast-biased codons and was named h_CYP2C9. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpUraADH2S/CYP2C9_yc was linearized at the *Stu*I site (as indicated by the arrow in Figure 4.58). Alternatively, the *Eco*RV or *Stu*I restriction sites could have been used for linearization.

The newly constructed plasmid YIpUraADH2S/CYP2C9 was further analysed via double digestion with *BamH*I, *Xba*I and digestion with only *Pvu*II restriction enzymes (Figure 4.56). The agarose gels show the expected sizes of DNA fragments.

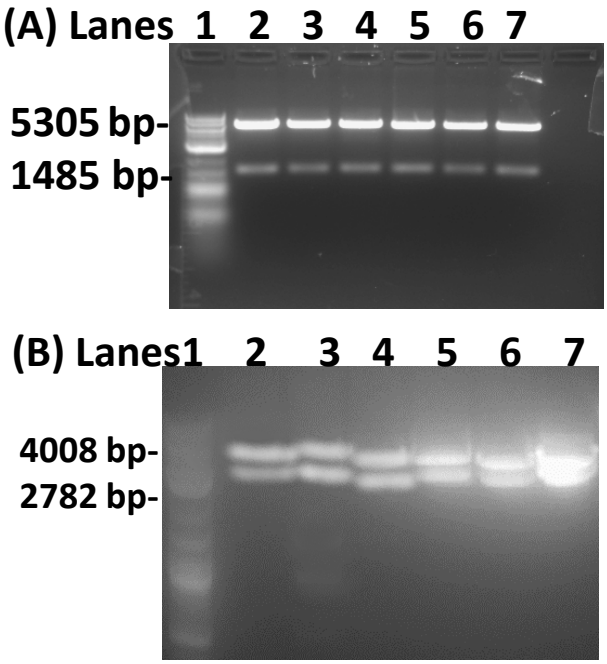


Figure 4.59. Agarose gels (A, B) that show the expected DNA fragments of YIpUraADH2S/CYP2C9_yc when plasmids isolated from seven bacterial clones were digested with BamHI-XbaI (lanes 2-7) and PvuII (lanes 2-7). Lane 1 (A & B), 2-log DNA ladder.

The plasmid YIpUraADH2S/CYP2C9_yc was used for expression of the *CYP2C9_yc* gene driven by the *ADH2* promoter. In order to allow this, the expression cassette *for CYP2C9_yc* gene, coding for the human CYP2C9 protein, was integrated at the *URA3* locus on chromosome V of yeast strains.

4.10.2 Construction of a yeast integrative plasmid that would allow stable expression of human CYP2C9 enzyme from the HIS3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpHisADH2S/CYP2C9_yc was created via the following steps:

(1) After digestion of the plasmid YIpHisADH2S (Figure 4.11) with restriction enzymes *BamH*I, *Xba*I, the 5305 bp vector fragment was isolated.

(2) A 1485 bp *BamH*I-*Xba*I *CYP2C9_yc* gene fragment was isolated. The gene was synthesized (Genewiz) as in Section 4.8.1, using yeast-biased codons based, based on the human protein sequence (NCBI Accession Number AAI25055).

(3) The 5305 bp vector and the 1485 bp insert were ligated to create the plasmid YIpHisADH2S/CYP2C9_yc (Figure 4.60).

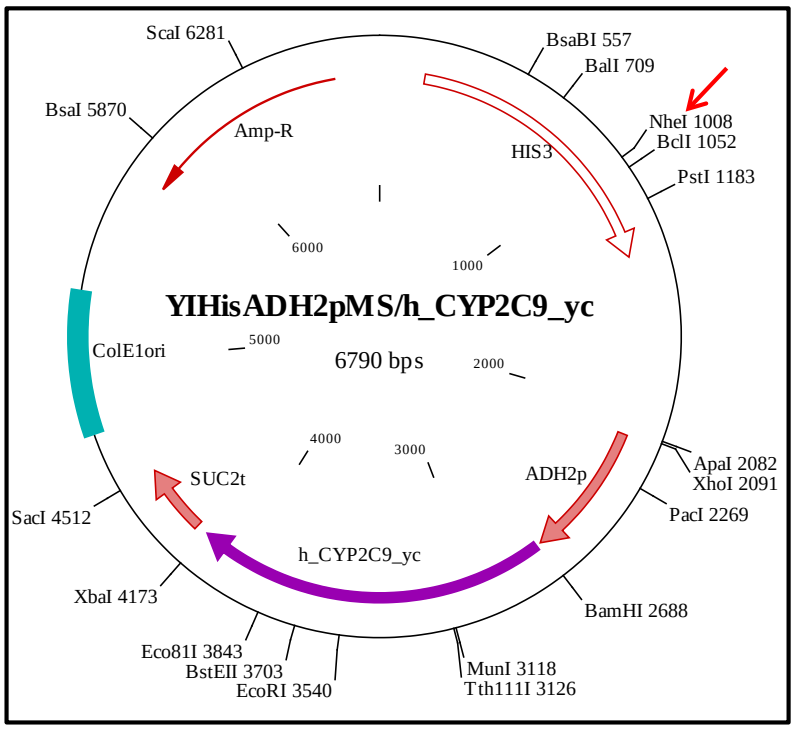


Figure 4.60. Map of plasmid YIpHisADH2S/CYP2C9_yc that allows integration of a human CYP2C9 gene expression cassette at the HIS3 locus of the yeast genome. The human CYP2C9 gene was synthesized using yeast-biased codons and was named h_CYP2C9. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpHisADH2S/CYP2C9_yc was linearized at the *Nhe*I site (indicated by the arrow in Figure 4.60). In order to facilitate homologous recombination, the restriction sites *Bal*I, *BsaB*I or *Bcl*I (Figure 4.60) could also have been used for linearization.

The newly constructed plasmid YIpHisADH2S/CYP2C9_yc was further analysed via double digestion with *BamH*I, XbaI and digestion with *Pvu*II (Figure 4.61). The agarose gels show the expected sizes of DNA fragments. The plasmid was used for expression of the *CYP2C9_yc* gene driven by the *ADH2* promoter. To allow this, the *CYP2C9_yc* gene expression cassette was integrated into the *HIS3* locus on chromosome XV.

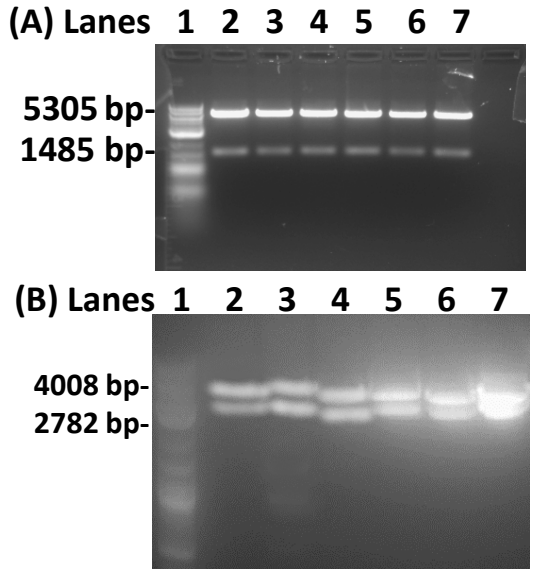


Figure 4.61. Agarose gels (A, B) that show the expected DNA fragments of YIpHisADH2S/CYP2C9_yc when plasmids isolated from seven bacterial clones were digested with BamHI-XbaI (lanes 2-7) and PvuII (lanes 2-7). Lane 1 (A & B), 2-log DNA ladder.

4.10.3 Construction of yeast strains that contain a copy of the CYP2C9_yc gene expression cassette integrated into two different chromosomal loci of the yeast strain YAB79

Two yeast strains, containing a single copy of the *CYP2C9_yc* gene expression cassette, were obtained by individually integrating the plasmids that bear the *CYP2C9_yc* expression cassette in the yeast strain YAB79 (BC300::ΔhRDM/LEU2$^+$, b5/TRP1$^+$; Figure 4.26) that expresses ΔhRDM from the *LEU2* locus and cytochrome b5 from the *TRP1* locus.

The two *CYP2C9_yc* gene encoding plasmids that were used for integration in the YAB79 strain were:

(1) YIpUraADH2S/CYP2C9_yc, and

(2) YIpHisADH2S/CYP2C9_yc.

After integration, the strains were named:

(a) YAB79::2C9_yc(URA3) and

(b) YAB79::2C9_yc(HIS3).

The amounts of enzyme expressed from these strains were then compared in fluorescence assays using an appropriate fluorogenic substrate. Comparative kinetic analysis of CY2C9 enzyme co-expressed with cytochrome b5 and cytochrome P450 reductase ΔhRDM, using 7-MFC as a substrate, is shown in Figure 4.62. The intact yeast cells bearing the *CYP2C9_yc* gene integrated in the chromosomal loci *HIS3* and *URA3*, the episomal plasmid bearing *CYP2C9_yc* gene and the control empty plasmid containing yeast strain were grown as described in Section 4.1.1. Comparative enzyme kinetic assays were performed as described in Section 4.1.2 to measure the formation of the product, HFC. These procedures were carried out in three different experiments

4.10.4 Comparison of activities of CYP2C9 enzyme expressed from (a) the CYP2C9_yc gene chromosomally integrated at the HIS3 or URA3 locus, and (b) an episomal plasmid bearing the CYP2C9_yc gene, in the yeast strain YAB79

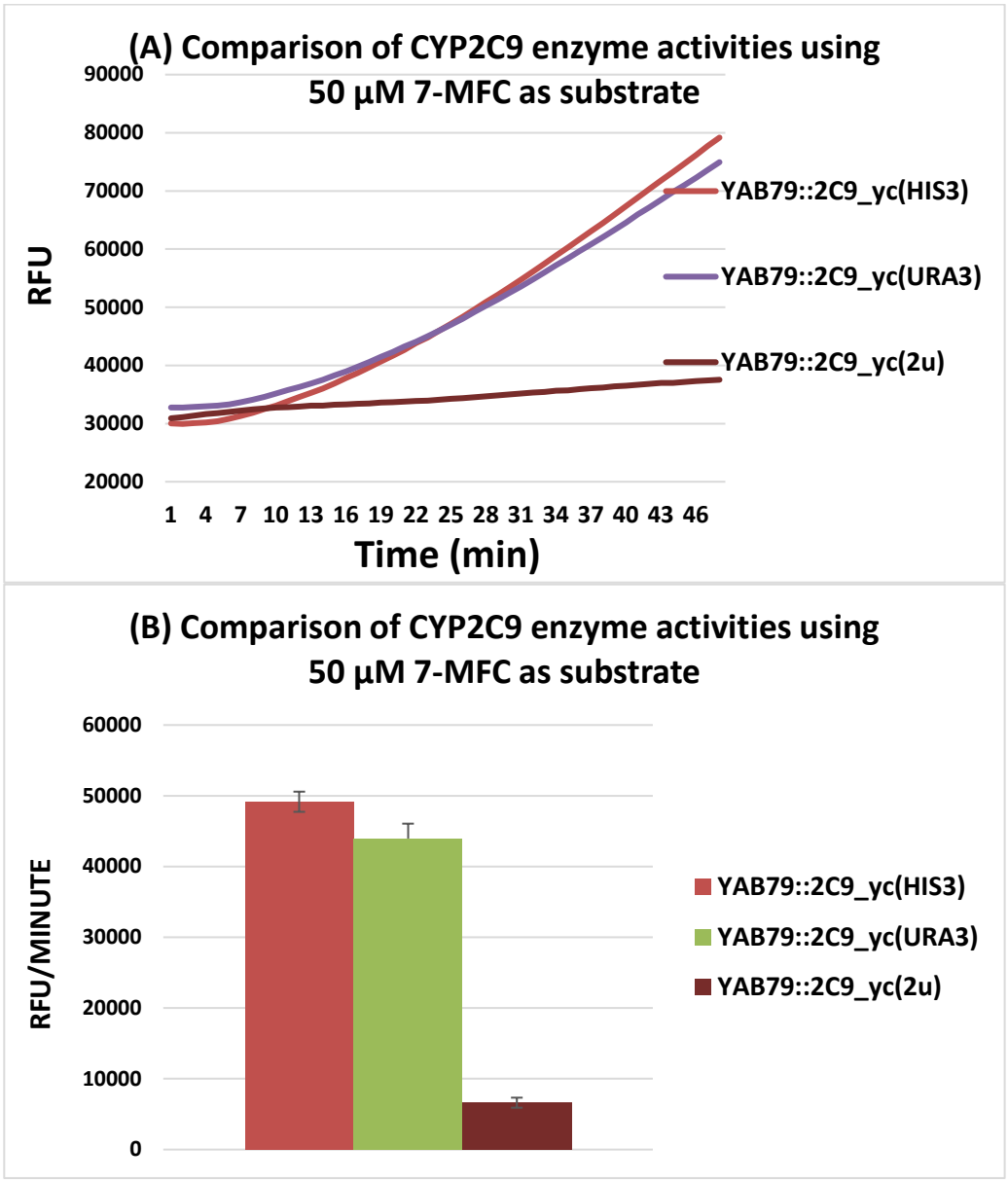


Figure 4.62. The graph (A) compares the CYP2C9 enzyme activities produced in the strains YAB79::2C9(HIS3) and YAB79::2C9(URA3), expressing a copy of the CYP2C9_yc gene expression cassette, integrated at the HIS3 and URA3 chromosomal loci, and the strain YAB79::2C9(2µ) expressing CYP2C9 from an episomal, 2-micron (2u) plasmid. The graphs represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

Figure 4.62 shows that the expression of a single copy of the *CYP2C9_yc* gene from the *HIS3* locus is slightly superior to that from the *URA3* locus, i.e. slightly more CYP2C9 enzyme is expressed from the *HIS3* locus than from the *URA3* locus The episomal plasmid distinctly provides much less enzyme, per unit number of cells, than the cells which have integrated copies of the *CYP2C9_yc* gene.

4.11 Construction of yeast strains, bearing expression cassettes of the human CYP4F3A gene, chemically synthesized using yeast biased codons, and comparison of CYP4F3A enzyme activities produced by different strains

4.11.1 Construction of a yeast integrative plasmid that would allow stable expression of human CYP4F3A enzyme from the HIS3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpHisADH2S/CYP4F3A_yc was created via the following steps:

(1) After digestion of the plasmid YIpHisADH2S (Figure 4.11) with restriction enzymes *BamH*I, *Xba*I, the 5305 bp vector fragment was isolated.

(2) Isolation of a 1587 bp *BamH*I-*Xba*I *CYP4F3A_yc* gene fragment. The gene was synthesized (Genewiz) using yeast-biased codons based on the protein template with NCBI Accession Number, NP_000887. The gene was isolated from a pUC57

based plasmid into which the chemically synthesised gene had originally been cloned.

(3) The 5305 bp vector and the 1587 bp insert were ligated to create the plasmid YIpHisADH2S/CYP4F3A_yc (Figure 4.62).

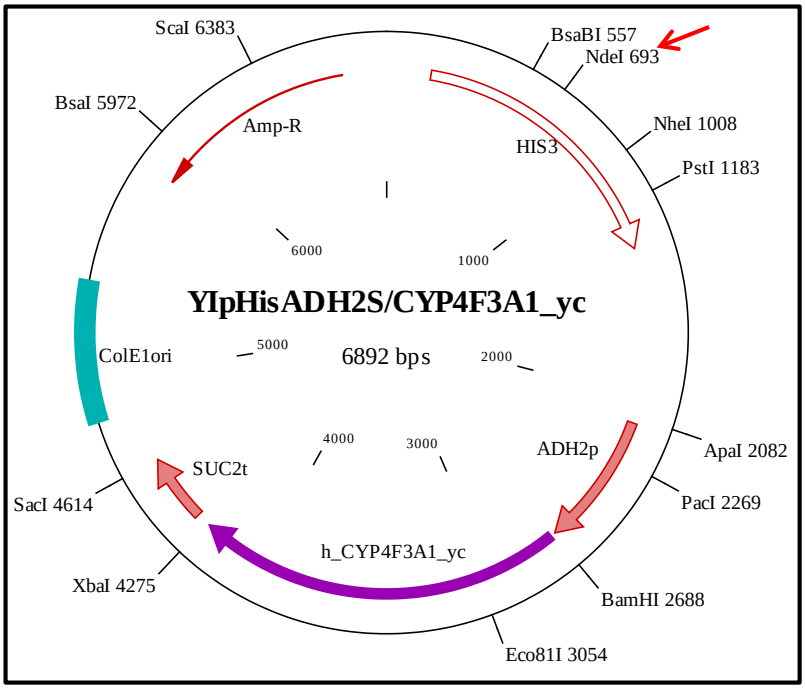


Figure 4.62. Map of plasmid YIpHisADH2S/CYP4F3A_yc that allows integration of a human *CYP4F3A* gene expression cassette at the *HIS3* locus of the yeast genome. The human *CYP4F3A* gene was synthesized using yeast-biased codons and was named h_CYP4F3A_yc. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpHisADH2S/CYP4F3A_yc was linearized at the *Nde*I site (indicated by the arrow in Figure 4.62). In order to facilitate homologous recombination, the restriction sites *BsaB*I or *Nhe*I (Figure 4.62) could also have been used for linearization.

The newly constructed plasmid YIpHisADH2S/CYP4F3A_yc was further analysed via double digestion with *BamH*I-XbaI restriction enzymes and a single digest with *NgoM*IV (Figure 4.63). The agarose gels show the expected sizes of DNA fragments.

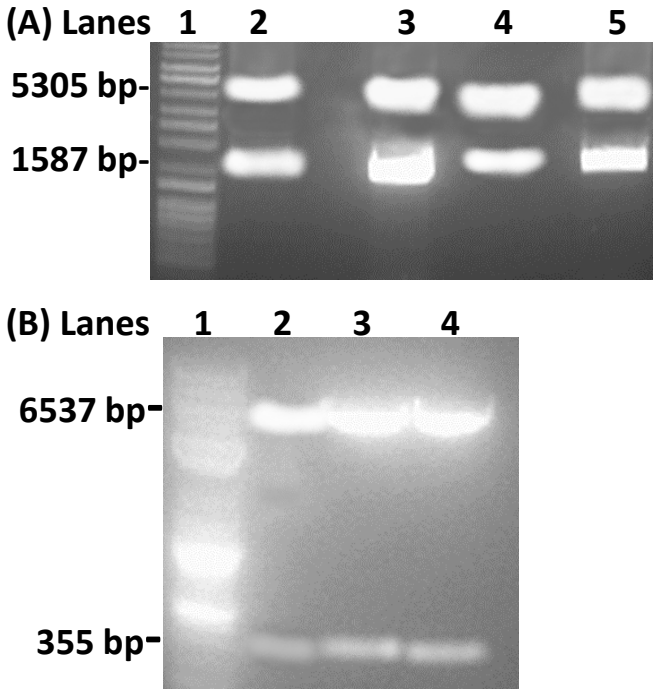


Figure 4.63. Agarose gels (A, B) that show the expected DNA fragments of YIpHisADH2S/CYP4F3A_yc when plasmids isolated from four (A) and three (B) bacterial clones were digested with BamHI-XbaI (lanes 2-5; A) and NgoMIV (lanes 2-4; B). Lane 1 (A & B), 2-log DNA ladder.

The YIpHisADH2S/CYP4F3A_yc plasmid can be used for expression of the *CYP4F3A_yc* gene driven by the *ADH2* promoter. In order to allow this, the *CYP4F3A_yc* gene expression cassette was integrated into the *HIS3* locus on chromosome XV.

4.11.2 Construction of a yeast integrative plasmid that would allow stable expression of human CYP4F3A enzyme from the URA3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpUraADH2S/CYP4F3A_yc [CYP4F3A_yc = gene coding for CYP4F3A protein (as in Section 4.9.1; Accession Number NP_000887) and synthesized using yeast-biased codons] was created via the following steps:

(1) After digestion of the vector YIpUraADH2S (Figure 4.15) with restriction enzymes *BamH*I, *Xba*I, a 5233 bp fragment was isolated. This eliminated 12 bp in the multi-cloning site.

(2) A 1587 bp *BamH*I-*Xba*I *CYP4F3A_yc* gene fragment was isolated, as in Section 4.9.1.

(3) The 5233 bp vector and the 1587 bp insert were ligated to obtain the plasmid YIpUraADH2S/CYP4F3A_yc (Figure 4.64).

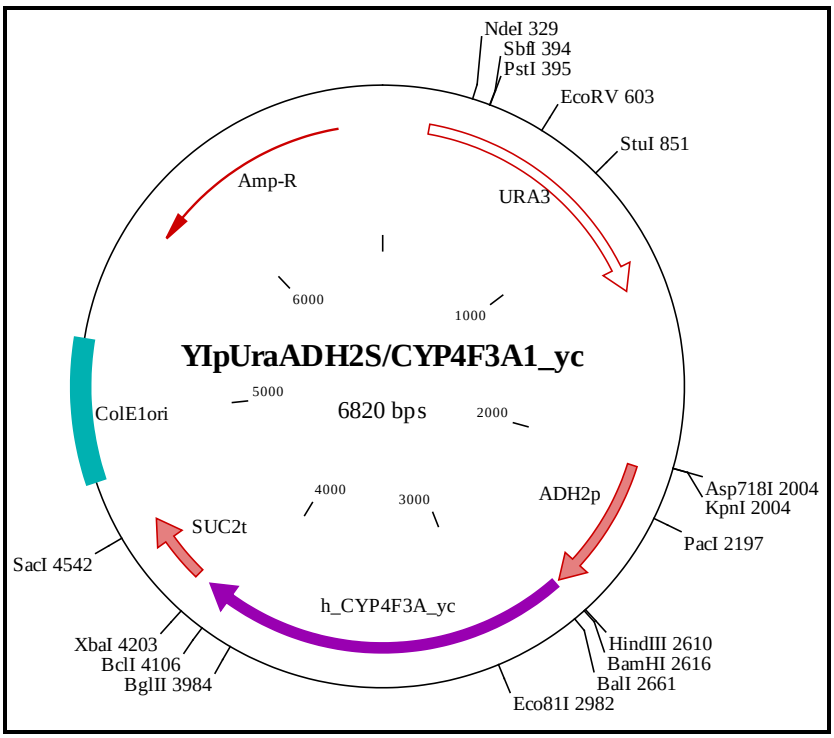


Figure 4.64. Map of plasmid YIpUraADH2S/CYP4F3A_yc that allows integration of a human *CYP4F3A* gene expression cassette at the URA3 locus of the yeast genome. The human *CYP4F3A* gene was synthesized using yeast-biased codons and was named h_CYP4F3A_yc. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpUraADH2S/CYP4F3A_yc was linearized at the *Stu*I site (as indicated by the arrow in Figure 4.64). Alternatively, the *Eco*RV or even *Pst*I site could have been used for linearization.

The newly constructed plasmid YIpUraADH2S/CYP4F3A_yc was further analysed via a single digest with *NgoM*IV (Figure 4.65). The agarose gel shows the expected sizes of the DNA fragments.

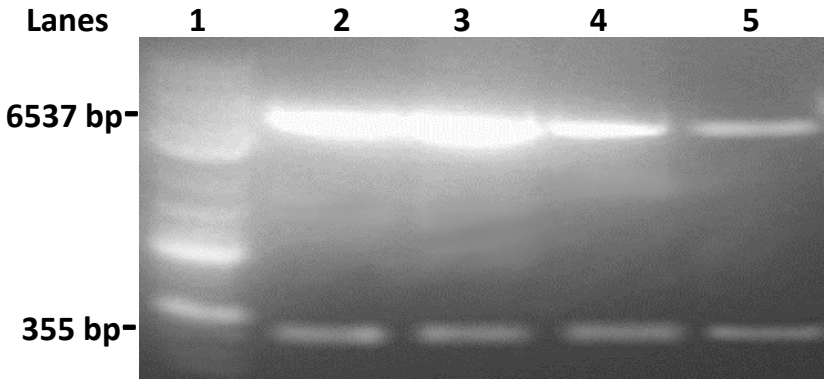


Figure 4.65. Agarose gel that shows the expected DNA fragments of YIpUraADH2S/CYP4F3A_yc when plasmids isolated from four bacterial clones were digested with NgoMIV (lanes 2-5). Lane 1, 2-log DNA ladder.

The plasmid YIpUraADH2S/CYP4F3A_yc was used for expression of the *CYP4F3A_yc* gene driven by the *ADH2* promoter. In order to allow this, the *CYP4F3A_yc* gene expression cassette was integrated into the *URA3* locus on chromosome V.

4.11.3 Construction of yeast strains that contain a copy of the CYP4F3A_yc gene expression cassette integrated into two different chromosomal loci of the yeast strain YAB79

Two yeast strains, containing a single copy of the *CYP4F3A_yc* gene expression cassette, were obtained by individually integrating the plasmids that bear the *CYP4F3A_yc* expression cassette in the yeast strain YAB79 (BC300::ΔhRDM/LEU2$^+$, b5/TRP1$^+$; Figure 4.26) that expresses ΔhRDM from the *LEU2* locus and cytochrome b5 from the *TRP1* locus.

The two *CYP4F3A_yc* gene encoding plasmids that were used for integration in the YAB79 strain were:

(1) YIpUraADH2S/CYP4F3A_yc, and

(2) YIpHisADH2S/CYP4F3A_yc.

After integration, the strains were named:

(a) YAB79::4F3A_yc(URA3) and

(b) YAB79::4F3A_yc(HIS3).

The amounts of enzyme expressed from these strains were then compared in fluorescence assays using an appropriate fluorogenic substrate. Comparative kinetic analysis of CY4F3A enzyme co-expressed with cytochrome b5 and cytochrome P450 reductase ΔhRDM, using CEC as a substrate, is shown in Figure 4.66. The intact yeast cells bearing the *CYP4F3A_yc* gene integrated in the chromosomal loci *HIS3* and *URA3*, the episomal plasmid bearing the *CYP4F3A_yc* gene and the control empty plasmid yeast strain were grown as described in Section 4.1.1. Comparative enzyme kinetic assays were performed, as described in Section 4.1.2, to measure the formation of the product, CHC. These procedures were carried out in three independent experiments.

4.11.4 Comparison of activities of CYP4F3A enzyme expressed from (a) the CYP4F3A_yc gene chromosomally integrated at the HIS3 or URA3 locus, and (b) an episomal plasmid bearing the CYP4F3A_yc gene, in the yeast strain YAB79

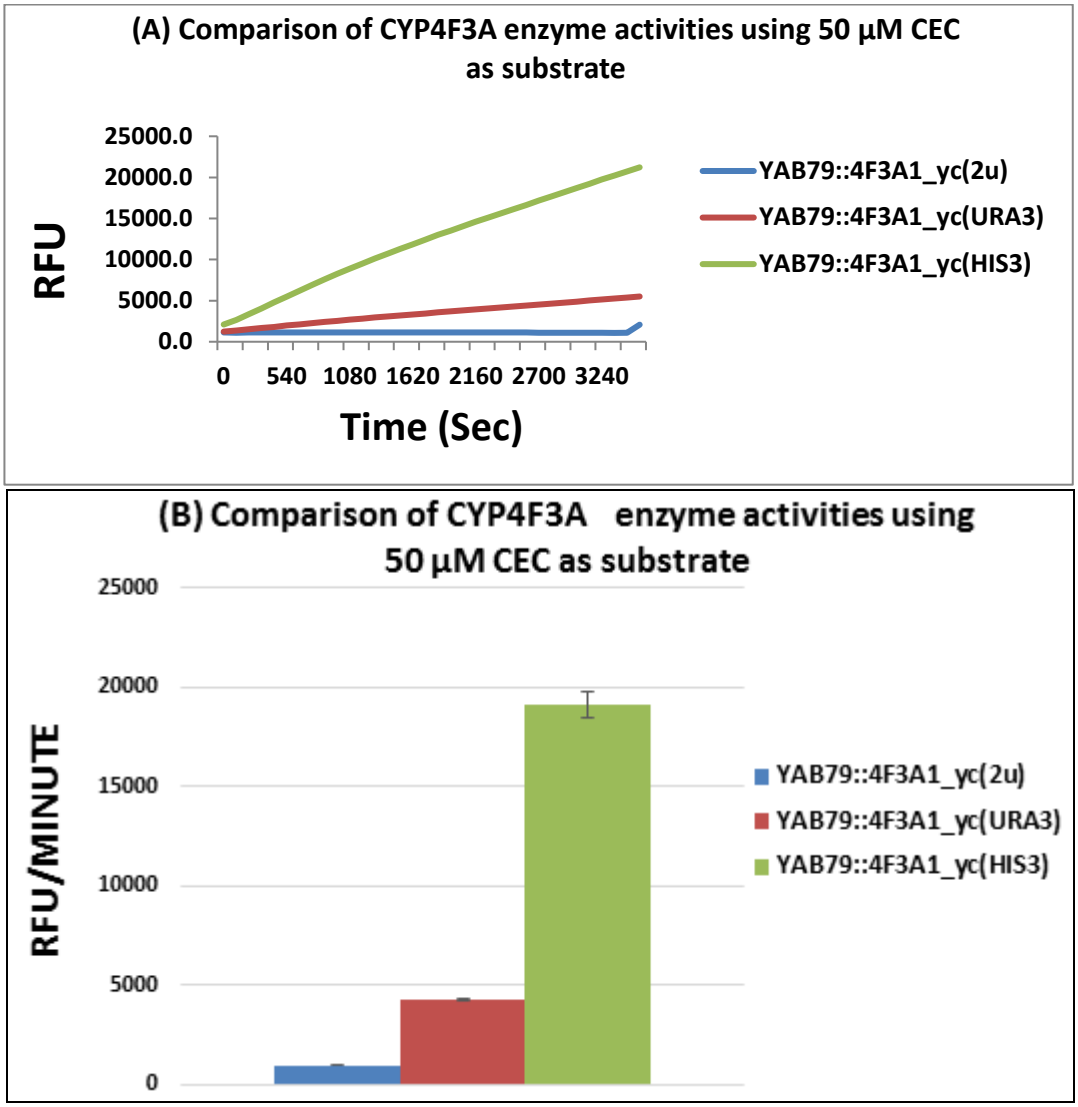


Figure 4.66. The graph (A) compares the CYP4F3A enzyme activities produced in the strains YAB79::4F3A(HIS3) and YAB79:: 4F3A(URA3), expressing a copy of the CYP4F3A_yc gene expression cassette the HIS3 and URA3 chromosomal loci, and the strain YAB79:: 4F3A(2µ) expressing CYP4F3A from an episomal, 2-micron (2u) plasmid. The graphs represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

Figure 4.66 clearly shows that the expression of a single copy of the *CYP4F3A_yc* gene in the yeast strain YAB79 from the *HIS3* chromosomal locus is far superior to expression from the *URA3* locus. Moreover, expression from the chromosomal *URA3* locus is > 3-fold better than the expression of CYP4F3A enzyme from an episomal, 2µ-plasmid which is an extra-chromosomal entity. Further corroboration of these results is provided in Chapter 5.

4.12 Construction of yeast strains, bearing expression cassettes of the human CYP1A1 gene, chemically synthesized using yeast biased codons, and comparison of CYP1A1 enzyme activities produced by different strains

4.12.1 Construction of a yeast integrative plasmid that would allow stable expression of human CYP1A1 enzyme from the HIS3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpHisADH2S/CYP1A1_yc was created via the following steps:

(1) After digestion of the plasmid YIpHisADH2S (Figure 4.11) with restriction enzymes *BamH*I, *Xba*I, the 5305 bp vector fragment was isolated.

(2) Isolation of a 1563 bp *BamH*I-*Xba*I *CYP1A1_yc* gene fragment. The gene was synthesized (Genewiz) using yeast-biased codons based on the protein template with NCBI Accession Number, NM_000499. The gene was isolated from a

pUC57 based plasmid into which the chemically synthesised gene had originally been cloned.

(3) The 5305 bp vector and the 1563 bp insert were ligated to create the plasmid YIpHisADH2S/CYP1A1_yc (Figure 4.67).

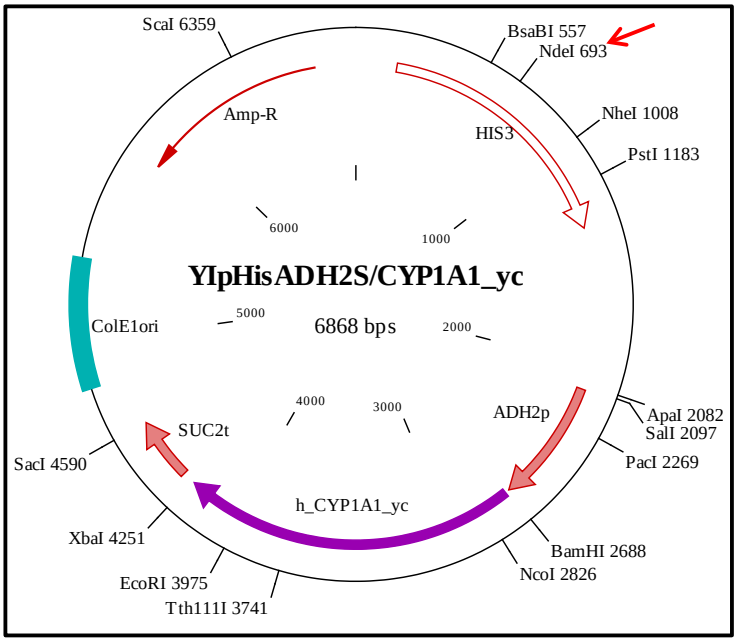


Figure 4.67. Map of plasmid YIpHisADH2S/CYP1A1_yc that allows integration of a human CYP1A1 gene expression cassette at the HIS3 locus of the yeast genome. The human CYP1A1 gene was synthesized using yeast-biased codons and was named h_CYP1A1_yc. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpHisADH2S/CYP1A1_yc was linearized at the *Nde*I site (indicated by the arrow in Figure 4.67). In order to facilitate homologous recombination, the restriction sites *BsaB*I or *Nhe*I (Figure 4.67) could also have been used for linearization.

The newly constructed plasmid YIpHisADH2S/CYP1A1_yc was further analysed via double digestion with *BamH*I-XbaI restriction enzymes and a single digestion with *Xho*I (Figure 4.68). The agarose gels show the expected sizes of DNA fragments.

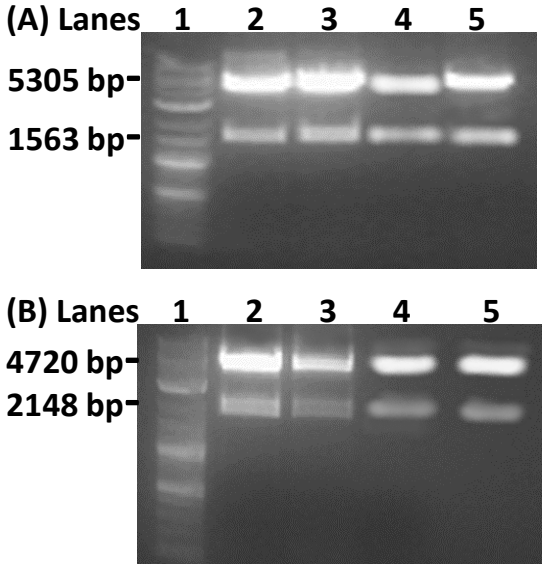


Figure 4.68. Agarose gels (A, B) that show the expected DNA fragments of YIpHisADH2S/CYP1A1_yc when plasmids isolated from four bacterial clones were digested with BamHI-XbaI (lanes 2-5; A) and XhoI (lanes 2-5; B). Lane 1 (A & B), 2-log DNA ladder.

The YIpHisADH2S/CYP1A1_yc plasmid can be used for expression of the *CYP1A1_yc* gene driven by the *ADH2* promoter. In order to allow this, the *CYP1A1_yc* gene expression cassette was integrated into the *HIS3* locus on chromosome XV.

4.12.2 Construction of a yeast integrative plasmid that would allow stable expression of human CYP1A1 enzyme from the URA3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpUraADH2S/CYP1A1_yc [CYP1A1_yc = gene coding for CYP1A1 protein (as in Section 4.10.1; Accession Number NM_000499) and synthesized using yeast-biased codons] was created via the following steps:

(1) After digestion of the vector YIpUraADH2S (Figure 4.15) with restriction enzymes *BamH*I, *Xba*I, a 5233 bp fragment was isolated. This eliminated 12 bp in the multi-cloning site.

(2) A 1563 bp *BamH*I-*Xba*I *CYP1A1_yc* gene fragment was isolated, as in Section 4.10.1.

(3) The 5233 bp vector and the 1587 bp insert were ligated to obtain the plasmid YIpUraADH2S/CYP1A1_yc (Figure 4.69).

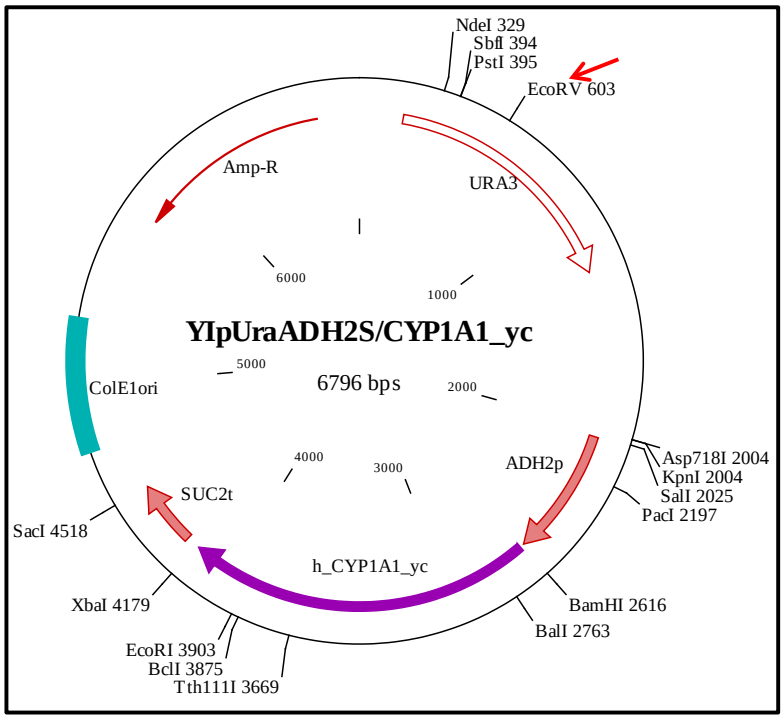


Figure 4.69. Map of plasmid YIpUraADH2S/CYP1A1_yc that allows integration of a human CYP1A1 gene expression cassette at the URA3 locus of the yeast genome. The human CYP1A1 gene was synthesized using yeast-biased codons and was named h_CYP1A1_yc. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpUraADH2S/CYP1A1_yc was linearized at the *Eco*RV site (as indicated by the arrow in Figure 4.69). Alternatively, the *Pst*I site could also be used for linearization.

The newly constructed plasmid YIpUraADH2S/CYP1A1_yc was further analysed via a digestions with *Pvu*II and *NgoM*IV (Figure 4.70). The agarose gels show the expected sizes of the DNA fragments.

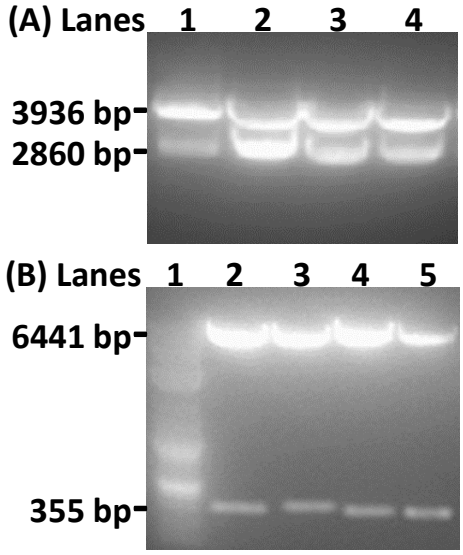


Figure 4.70. Agarose gels (A, B) that show the expected DNA fragments of YIpUraADH2S/CYP1A1_yc when plasmids isolated from four bacterial clones were digested with PvuII (lanes 1-4; A) and NgoMIV (lanes 2-5; B). Lane 1 (B), 2-log DNA ladder.

The plasmid YIpUraADH2S/CYP1A1_yc was used for expression of the *CYP1A1_yc* gene driven by the *ADH2* promoter. In order to allow this, the *CYP1A1_yc* gene expression cassette was integrated into the *URA3* locus on chromosome V.

4.12.3 Construction of yeast strains that contain a copy of the CYP1A1_yc gene expression cassette integrated into two different chromosomal loci of the yeast strain YY7

Two yeast strains, containing a single copy of the *CYP1A1_yc* gene expression cassette, were obtained by individually integrating the plasmids that bear the *CYP1A1_yc*

expression cassette in the yeast strain YY7 (BC300::ΔhRDM/LEU2^{+}; Figure 4.26) that expresses ΔhRDM from the *LEU2* locus.

The two *CYP1A1_yc* gene encoding plasmids that were used for integration in the YAB79 strain were:

(1) YIpUraADH2S/CYP1A1_yc, and

(2) YIpHisADH2S/CYP1A1_yc.

After integration, the strains were named:

(a) YY7::1A1_yc(URA3) and

(b) YY7::1A1_yc(HIS3).

The amounts of enzyme expressed from these strains were then compared in fluorescence assays using an appropriate fluorogenic substrate. Comparative kinetic analysis of CY1A1 enzyme co-expressed without cytochrome b5 but in the presence of cytochrome P450 reductase ΔhRDM, using 7-ER as a substrate is shown in Figure 4.71. The intact yeast cells bearing the *CYP1A1_yc* gene integrated in the chromosomal loci *HIS3* and *URA3*, the episomal plasmid bearing the *CYP1A1_yc* gene and the control empty plasmid containing yeast strain were grown as described earlier. Comparative enzyme kinetic assays were performed to measure the formation of the product, resorufin, from 7-ER. The experiment was carried out three times.

4.12.4 Comparison of activities of CYP1A1 enzyme expressed from (a) the CYP1A1_yc gene chromosomally integrated at the HIS3 or URA3 locus, and (b) an episomal plasmid bearing the CYP1A1_yc gene, in the yeast strain YY7

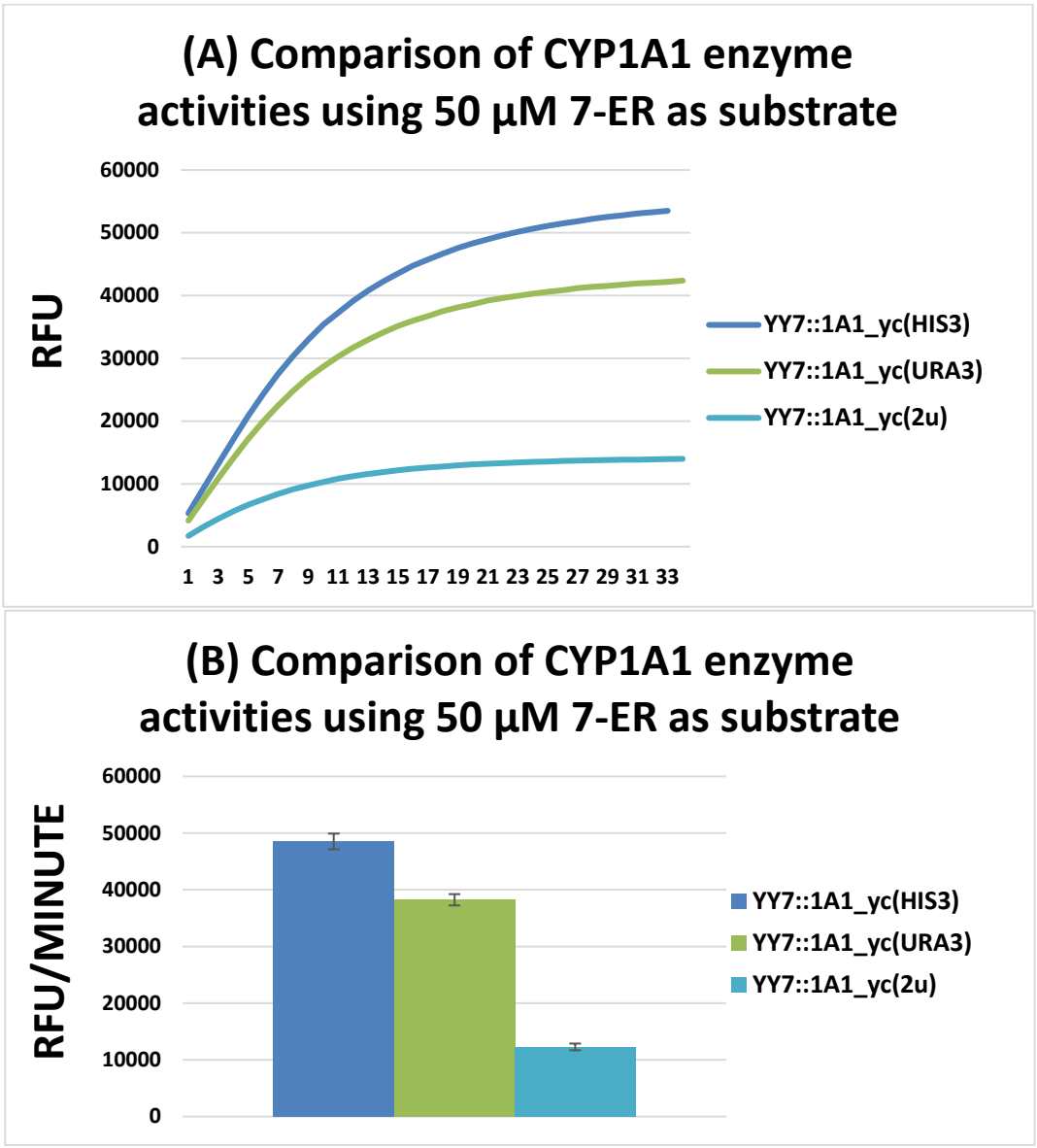


Figure 4.71. The graph (A) compares the CYP1A1 enzyme activities produced in the strains YY7::1A1(HIS3) and YY7::1A1(URA3), expressing a copy of the CYP1A1_yc gene expression cassette, integrated at the HIS3 and URA3 chromosomal loci, and the strain YY7::1A1(2µ) expressing CYP1A1 from an episomal, 2-micron (2u) plasmid. The graphs represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

Figure 4.71 shows the expression of a single copy of the *CYP1A1_yc* gene in the baker's yeast strain YY7 from the *HIS3* and *URA3* chromosomal loci. It could be inferred that CYP1A1 enzyme is expressed at higher levels from the *HIS3* locus than from the *URA3* locus while the enzyme levels produced from the *URA3* locus was higher than that obtained from a strain that expressed *CYP1A1_yc* from an episomal plasmid. Further corroboration of these results is provided in Chapter 5 of this study.

The results suggest that the amounts of CYP1A1 expressed depend on the genetic locus and the chromosome in which the CYP1A1_yc gene expression cassette is integrated.

4.13 Construction of yeast strains, bearing expression cassettes of the human CYP1B1 gene, chemically synthesized using yeast biased codons, and comparison of CYP1B1 enzyme activities produced by different strains

4.13.1 Construction of a yeast integrative plasmid that would allow stable expression of human CYP1B1 enzyme from the HIS3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpHisADH2S/CYP1B1_yc was created via the following steps:

(1) After digestion of the plasmid YIpHisADH2S (Figure 4.11) with restriction enzymes *BamH*I, *Xba*I, the 5305 bp vector fragment was isolated.

(2) Isolation of a 1656 bp *BamH*I-*Xba*I *CYP1B1_yc* gene fragment. The gene was synthesized (Genewiz) using yeast-biased codons based on the protein template with NCBI Accession Number, NM_000104. The gene was isolated from a pUC57 based plasmid into which the chemically synthesised gene had originally been cloned.

(3) The 5305 bp vector and the 1656 bp insert were ligated to create the plasmid YIpHisADH2S/CYP1B1_yc (Figure 4.72).

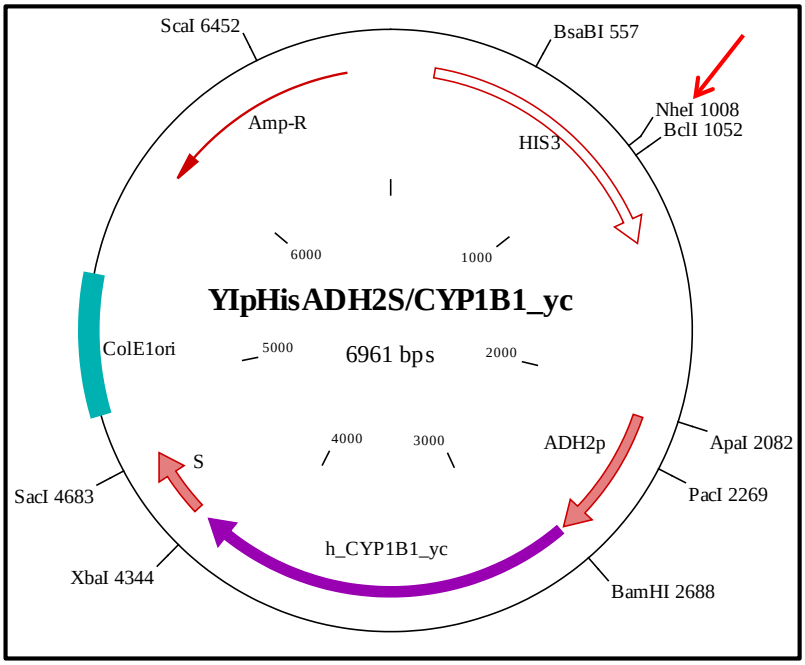


Figure 4.72. Map of plasmid YIpHisADH2S/CYP1B1_yc that allows integration of a human CYP1B1 gene expression cassette at the HIS3 locus of the yeast genome. The human CYP1B1 gene was synthesized using yeast-biased codons and was named h_CYP1B1_yc. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpHisADH2S/CYP1B1_yc was linearized at the *Nhe*I site (indicated by the arrow in Figure 4.72). In order to facilitate homologous recombination, the restriction sites *BsaB*I or *Bcl*I (Figure 4.72) could also have been used for linearization.

The newly constructed plasmid YIpHisADH2S/CYP1B1_yc was further analysed via double digestion with *BamH*I-XbaI restriction enzymes and a single digestion with *Xho*I (Figure 4.73). The agarose gels show the expected sizes of DNA fragments.

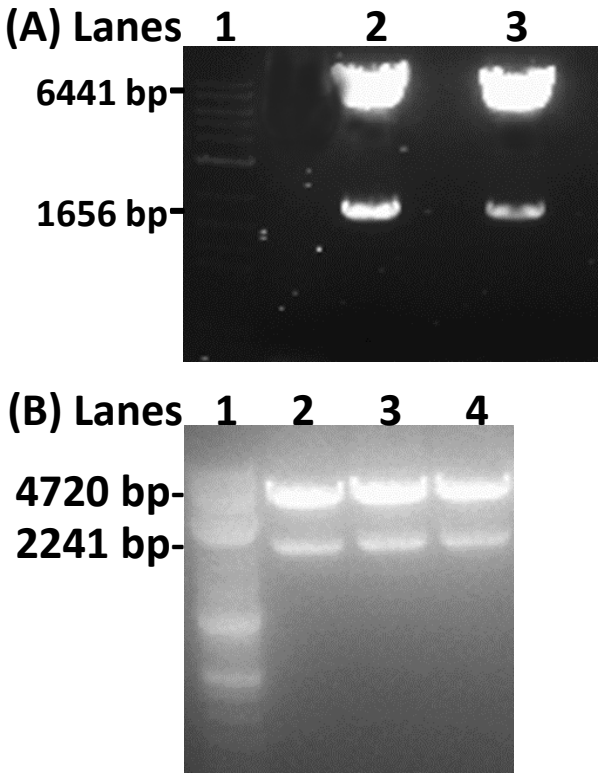


Figure 4.73. Agarose gels (A, B) that show the expected DNA fragments of YIpHisADH2S/CYP1B1_yc when plasmids isolated from two (A) and three (B) bacterial clones were digested with BamHI-XbaI (lanes 2-3; A) and XhoI (lanes 2-4; B). Lane 1 (A & B), 2-log DNA ladder.

The YIpHisADH2S/CYP1B1_yc plasmid can be used for expression of the *CYP1B1_yc* gene driven by the *ADH2* promoter. In order to allow this, the *CYP1B1_yc* gene expression cassette was integrated into the *HIS3* locus on chromosome XV.

4.13.2 Construction of a yeast integrative plasmid that would allow stable expression of human CYP1B1 enzyme from the URA3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpUraADH2S/CYP1B1_yc [CYP1B1_yc = gene coding for CYP1B1 protein (as in Section 4.11.1; Accession Number NM_000104) and synthesized using yeast-biased codons] was created via the following steps:

(1) After digestion of the vector YIpUraADH2S (Figure 4.15) with restriction enzymes *BamH*I, *Xba*I, a 5233 bp fragment was isolated. This eliminated 12 bp in the multi-cloning site.

(2) A 1656 bp *BamH*I-*Xba*I *CYP1B1_yc* gene fragment was isolated, as in Section 4.11.1.

(3) The 5233 bp vector and the 1656 bp insert were ligated to obtain the plasmid YIpUraADH2S/CYP1B1_yc (Figure 4.74).

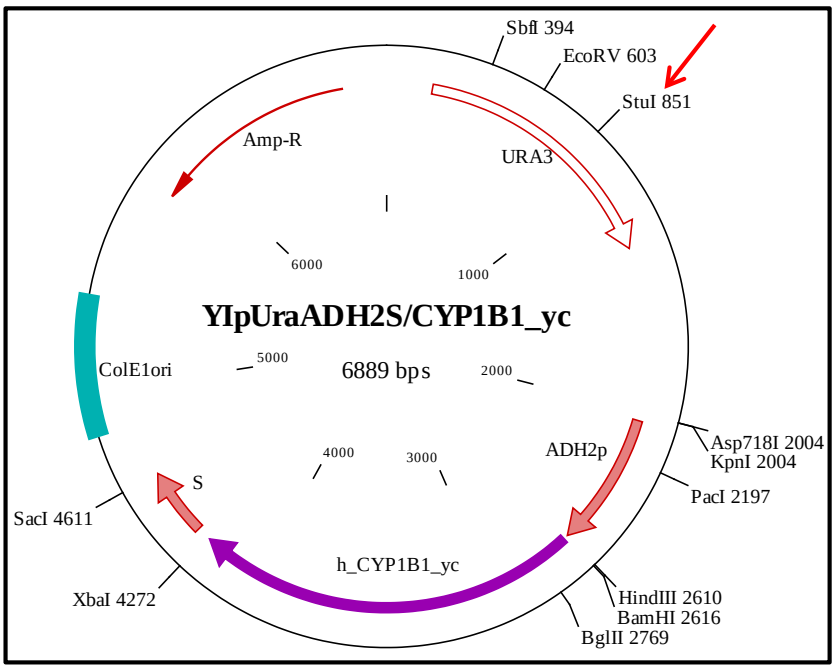


Figure 4.74. Map of plasmid YIpUraADH2S/CYP1B1_yc that allows integration of a human CYP1B1 gene expression cassette at the URA3 locus of the yeast genome. The human CYP1B1 gene was synthesized using yeast-biased codons and was named h_CYP1B1_yc. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpUraADH2S/CYP1B1_yc was linearized at the *Stu*I site (as indicated by the arrow in Figure 4.74). Alternatively, the *EcoR*V site could also be used for linearization.

The newly constructed plasmid YIpUraADH2S/CYP1B1_yc was further analysed via a double digestion with the restriction enzyme *BamH*I-*Xba*I (Figure 4.75). The agarose gel shows the expected sizes of the DNA fragments.

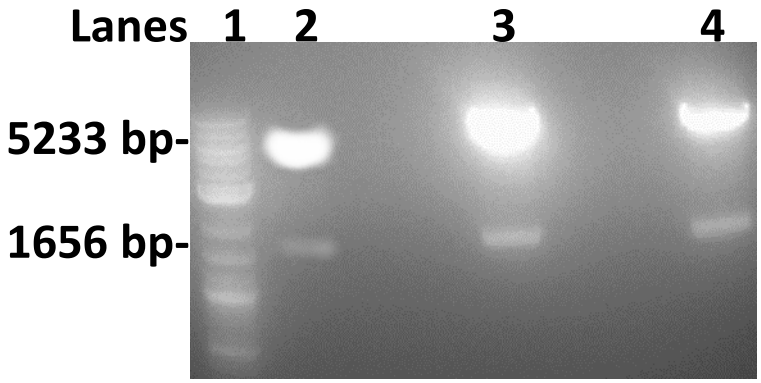


Figure 4.75. Agarose gel shows the expected DNA fragments of YIpUraADH2S/CYP1B1_yc when plasmids isolated from three bacterial clones were digested with BamHI-XbaI (lanes 2-3). Lane 1, 2-log DNA ladder.

The plasmid YIpUraADH2S/CYP1B1_yc was used for expression of the *CYP1B1_yc* gene driven by the *ADH2* promoter. In order to allow this, the *CYP1B1_yc* gene expression cassette was integrated into the *URA3* locus on chromosome V.

4.13.3 Construction of yeast strains that contain a copy of the CYP1B1_yc gene expression cassette integrated into two different chromosomal loci of the yeast strain YY7

Two yeast strains, containing a single copy of the *CYP1B1_yc* gene expression cassette, were obtained by individually integrating the plasmids that bear the *CYP1B1_yc* expression cassette in the yeast strain YY7 (BC300::ΔhRDM/LEU2$^+$; Figure 4.26) that expresses ΔhRDM from the *LEU2* locus.

The two *CYP1B1_yc* gene encoding plasmids that were used for integration in the YAB79 strain were:

(1) YIpUraADH2S/CYP1B1_yc, and

(2) YIpHisADH2S/CYP1B1_yc.

After integration, the strains were named:

(a) YY7::1B1_yc(URA3) and

(b) YY7::1B1_yc(HIS3).

The amounts of enzyme expressed from these strains were then compared in fluorescence assays using an appropriate fluorogenic substrate. The comparative kinetic analysis of CYP1B1 enzyme gene co-expressed with cytochrome P450 reductase ΔhRDM, in the absence of cytochrome b5, using 7-ER as a substrate is shown in Figure 4.76. The intact yeast cells bearing the *CYP1B1_yc* gene integrated at the chromosomal loci, *HIS3* or *URA3*, the episomal plasmid bearing the *CYP1B1_yc* gene and the control empty plasmid containing yeast strain were grown as described in Section 4.1.1. Comparative kinetic assays were performed, as described in Section 4.1.2 to measure formation of the product, resorufin. These assays were carried out in three independent experiments.

4.13.4 Comparison of activities of CYP1B1 enzyme expressed from (a) the CYP1B1_yc gene chromosomally integrated at the HIS3 or URA3 locus, and (b) an episomal plasmid bearing the CYP1B1_yc gene, in the yeast strain YY7

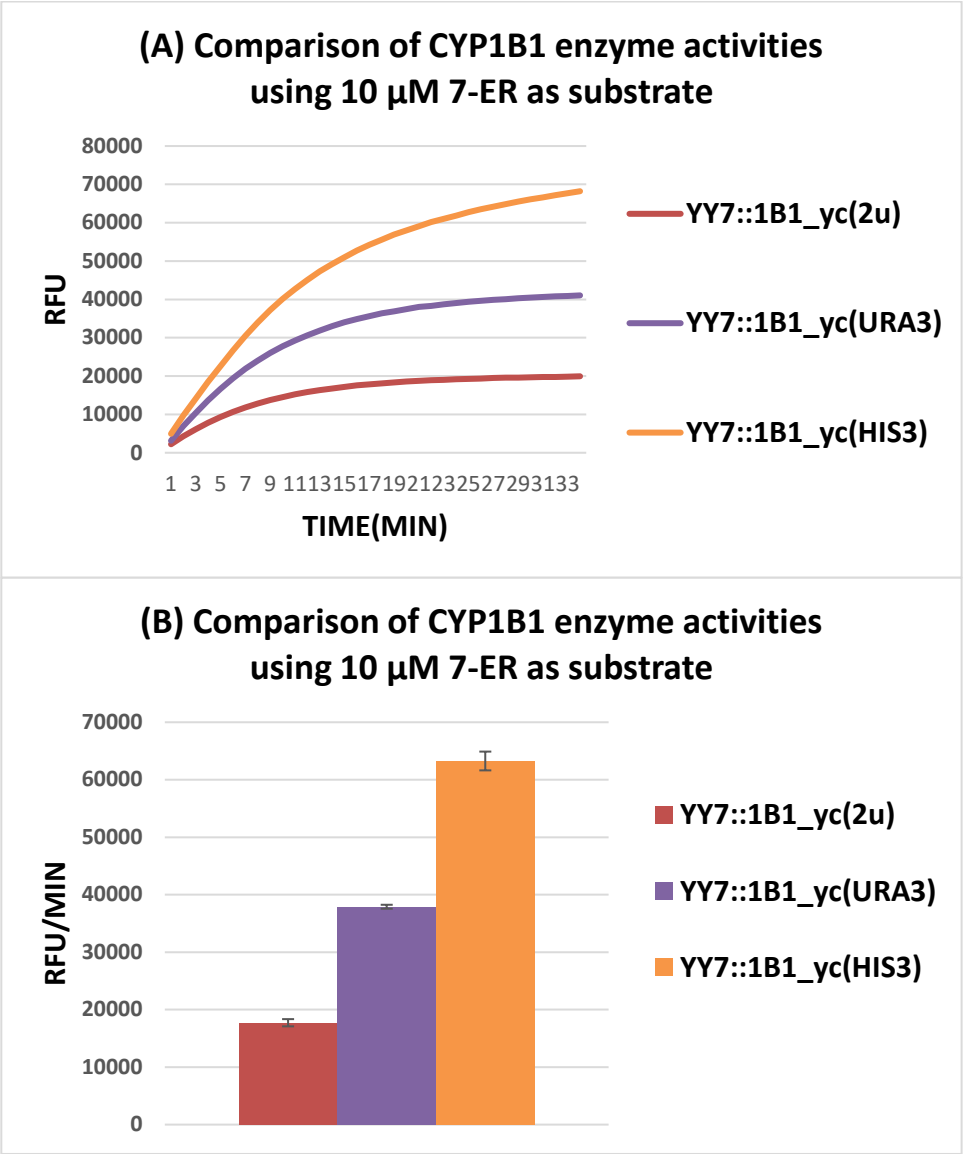


Figure 4.76. The graph (A) compares the CYP1B1 enzyme activities produced in the strains YY7::1A1(HIS3), YY7::1A1(URA3) and YY7::1A1(HIS3,URA3) with the strain YY7::1A1(2µ) expressing CYP1B1 from an episomal, 2-micron (2u) plasmid. The first two integrated strains contain a copy of the CYP1B1_yc gene expression cassette, integrated at the HIS3 and URA3 chromosomal loci, whereas the third integrated strain contains two copies of CYP1B1_yc. The graphs represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

The results show that the cytochrome P450 enzyme CYP1B1 is expressed at a much higher level from the *HIS3* locus than from the *URA3* locus, within live yeast cells. The *CYP1B1_yc* gene expressed from both chromosomal loci expresses more CYP1B1 enzyme than when it is expressed from an episomal plasmid. Further confirmation of these results is revealed in Chapter 5 of this study.

Figure 4.76 suggests that the amounts of CYP1B1 expressed depends on the genetic locus and the chromosome in which the *CYP1B1_yc* gene expression cassette is integrated.

4.14 Construction of yeast strains, bearing expression cassettes of the human CYP3A5 gene, chemically synthesized using yeast biased codons, and comparison of CYP3A5 enzyme activities produced by different strains

4.14.1 Construction of a yeast integrative plasmid that would allow stable expression of human CYP3A5 enzyme from the HIS3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpHisADH2S/CYP3A5_yc was created via the following steps:

(1) After digestion of the plasmid YIpHisADH2S (Figure 4.11) with restriction enzymes *BamH*I, *Xba*I, the 5305 bp vector fragment was isolated.

(2) Isolation of a 1533 bp *BamH*I-*Xba*I *CYP3A5_yc* gene fragment. The gene was synthesized (Genewiz) using yeast-biased codons based on the protein template with NCBI Accession Number, NP_000768. The gene was isolated from a pUC57 based plasmid into which the chemically synthesised gene had originally been cloned.

(3) The 5305 bp vector and the 1533 bp insert were ligated to create the plasmid YIpHisADH2S/CYP3A5_yc (Figure 4.77).

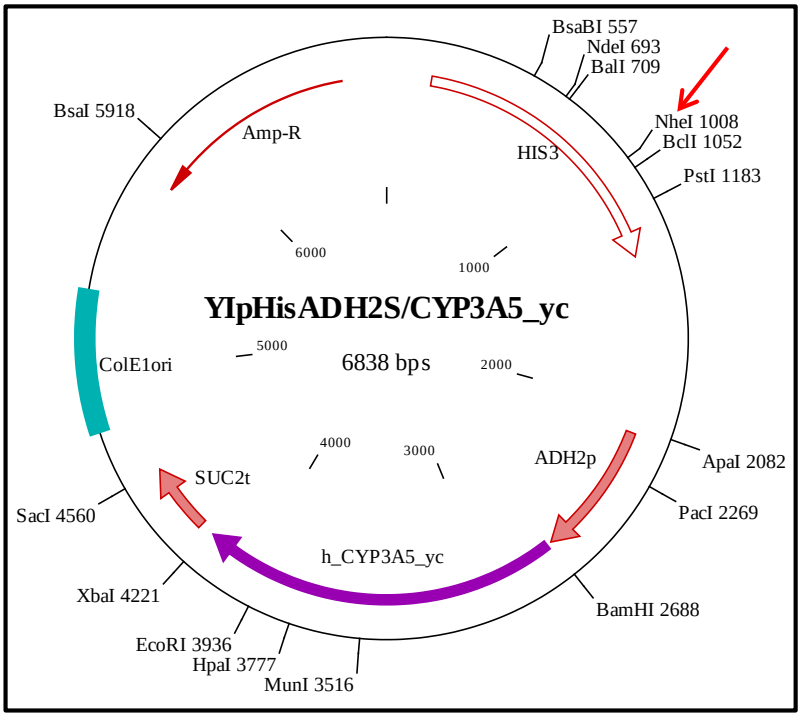


Figure 4.77. Map of plasmid YIpHisADH2S/CYP3A5_yc that allows integration of a human CYP3A5 gene expression cassette at the HIS3 locus of the yeast genome. The human CYP3A5 gene was synthesized using yeast-biased codons and was named h_CYP3A5_yc. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpHisADH2S/CYP3A5_yc was linearized at the *Nhe*I site (indicated by the arrow in

Figure 4.77). In order to facilitate homologous recombination, the restriction sites *BsaB*I, *Nde*I, *Bal*I or *Bcl*I (Figure 4.77) could also have been used for linearization.

The newly constructed plasmid YIpHisADH2S/CYP3A5_yc was further analysed via double digestion with *BamH*I-XbaI restriction enzymes and a single digestion with *Xho*I (Figure 4.78). The agarose gels show the expected sizes of DNA fragments.

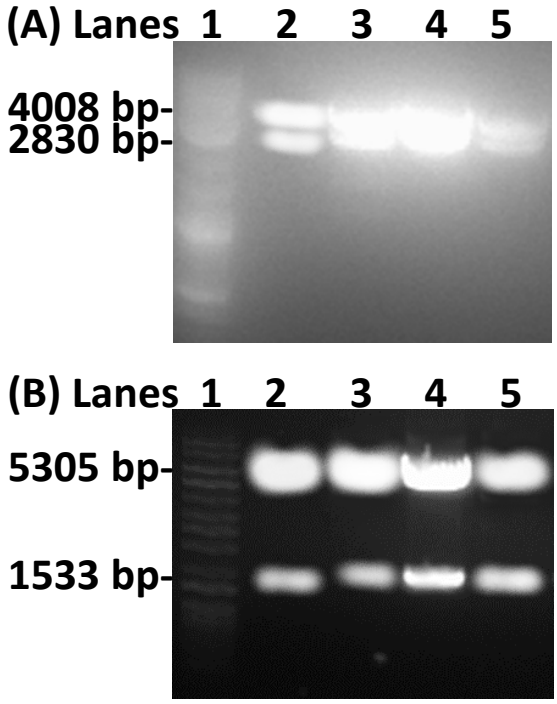


Figure 4.78. Agarose gels (A, B) that show the expected DNA fragments of YIpHisADH2S/CYP3A5_yc when plasmids isolated from four (A, B) bacterial clones were digested with PvuII (lanes 2-5; A) and BamHI-XbaI (lanes 2-5; B). Lane 1 (A & B), 2-log DNA ladder.

The YIpHisADH2S/CYP3A5_yc plasmid can be used for expression of the *CYP3A5_yc* gene driven by the *ADH2* promoter. In order to allow this, the *CYP3A5_yc* gene expression cassette was integrated into the *HIS3* locus on chromosome XV.

4.14.2 Construction of a yeast integrative plasmid that would allow stable expression of human CYP3A5 enzyme from the URA3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpUraADH2S/CYP3A5_yc [CYP3A5_yc = gene coding for CYP3A5 protein (as in Section 4.12.1; Accession Number NP_000768) and synthesized using yeast-biased codons] was created via the following steps:

(1) After digestion of the vector YIpUraADH2S (Figure 4.15) with restriction enzymes *BamH*I, *Xba*I, a 5233 bp fragment was isolated. This eliminated 12 bp in the multi-cloning site.

(2) A 1533 bp *BamH*I-*Xba*I *CYP3A5_yc* gene fragment was isolated, as in Section 4.12.1.

(3) The 5233 bp vector and the 1656 bp insert were ligated to obtain the plasmid YIpUraADH2S/CYP3A5_yc (Figure 4.79).

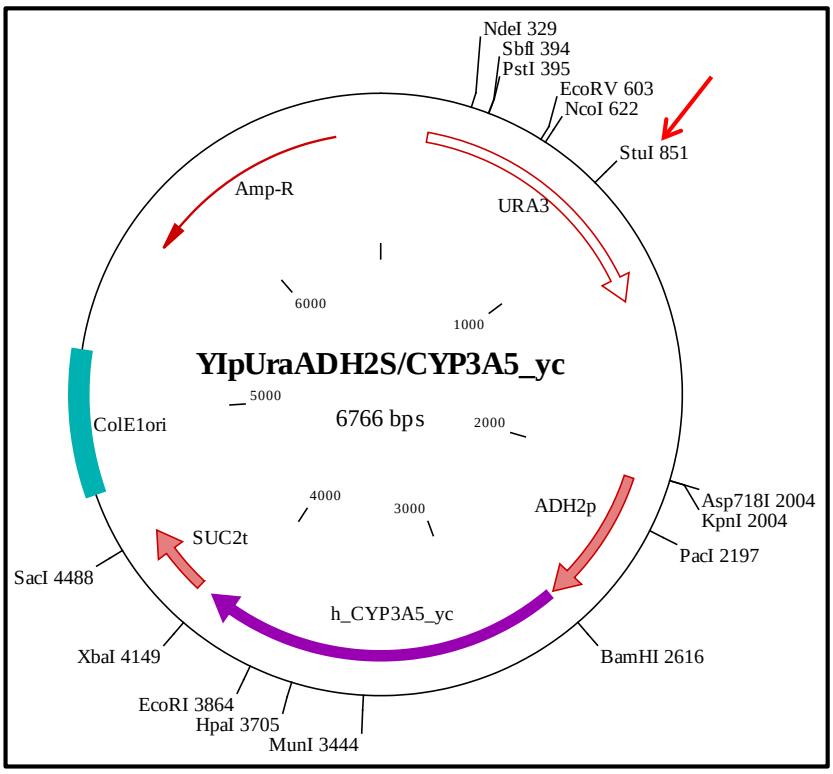


Figure 4.79. Map of plasmid YIpUraADH2S/CYP3A5_yc that allows integration of a human CYP3A5 gene expression cassette at the URA3 locus of the yeast genome. The human CYP3A5 gene was synthesized using yeast-biased codons and was named h_CYP3A5_yc. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpUraADH2S/CYP3A5_yc was linearized at the *Stu*I site (as indicated by the arrow in Figure 4.79). Alternatively, the *EcoR*V or *Nco*I site could also have been used for linearization.

The newly constructed plasmid YIpUraADH2S/CYP3A5_yc was further analysed via a digestions with *BamH*I-*Xba*I and *Pvu*II (Figure 4.80). The agarose gels show the expected sizes of the DNA fragments.

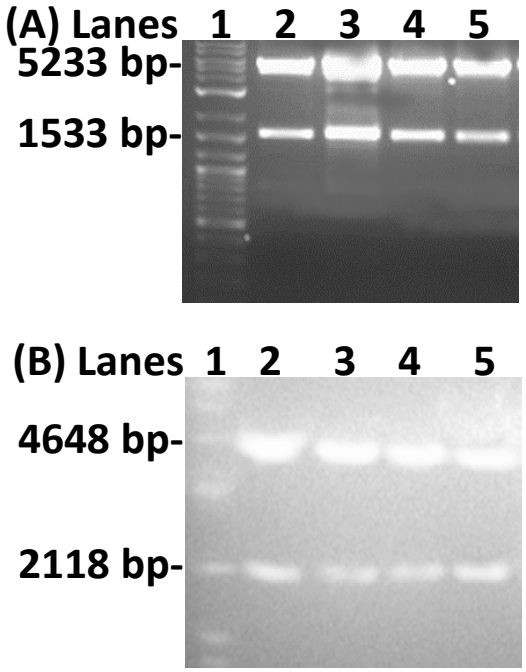


Figure 4.80. Agarose gels (A, B) that show the expected DNA fragments of YIpUraADH2S/CYP3A5_yc when plasmids isolated from four (A, B) bacterial clones were digested with BamHI-XbaI (lanes 2-5; A) and PvuII (lanes 2-5; B). Lane 1 (A & B), 2-log DNA ladder.

The plasmid YIpUraADH2S/CYP3A5_yc was used for expression of the *CYP3A5_yc* gene driven by the *ADH2* promoter. In order to allow this, the *CYP3A5_yc* gene expression cassette was integrated into the *URA3* locus on chromosome V.

4.14.3 Construction of yeast strains that contain a copy of the CYP3A5_yc gene expression cassette integrated into two different chromosomal loci of the yeast strain YAB79

Two yeast strains, containing a single copy of the *CYP3A5_yc* gene expression cassette, were obtained by individually integrating the plasmids that bear the *CYP3A5_yc* expression cassette in the yeast strain YAB79 (BC300::ΔhRDM/LEU2$^+$, b5/TRP1;

Figure 4.26) that expresses ΔhRDM from the *LEU2* locus and cytochrome b5 from the *TRP1* locus.

The two *CYP3A5_yc* gene encoding plasmids that were used for integration in the YAB79 strain were:

(1) YIpUraADH2S/CYP3A5_yc, and

(2) YIpHisADH2S/CYP3A5_yc.

After integration, the strains were named:

(a) YAB79::3A5_yc(URA3) and

(b) YAB79::3A5_yc(HIS3).

The amounts of enzyme expressed from these strains were then compared in fluorescence assays using an appropriate fluorogenic substrate. The comparative kinetic analysis of CYP3A5 enzyme co-expressed with cytochrome b5 and cytochrome P450 reductase ΔhRDM, using DBF as a substrate, is shown in Figure 4.81. The intact yeast cells bearing the *CYP3A5_yc* gene integrated in the chromosomal loci *HIS3* and *URA3*, the episomal plasmid bearing the *CYP3A5_yc* gene and the control empty plasmid containing yeast strain were grown as described in Section 4.1.1. The comparative enzyme kinetic assays were performed as described in Section 4.1.2 to measure formation of the product, fluoroscein. These procedures were carried out in three independent experiments.

4.14.4 Comparison of activities of CYP3A5enzyme expressed from (a) the CYP3A5_yc gene chromosomally integrated at the HIS3 or URA3 locus, and (b) an episomal plasmid bearing the CYP3A5_yc gene, in the yeast strain YAB79

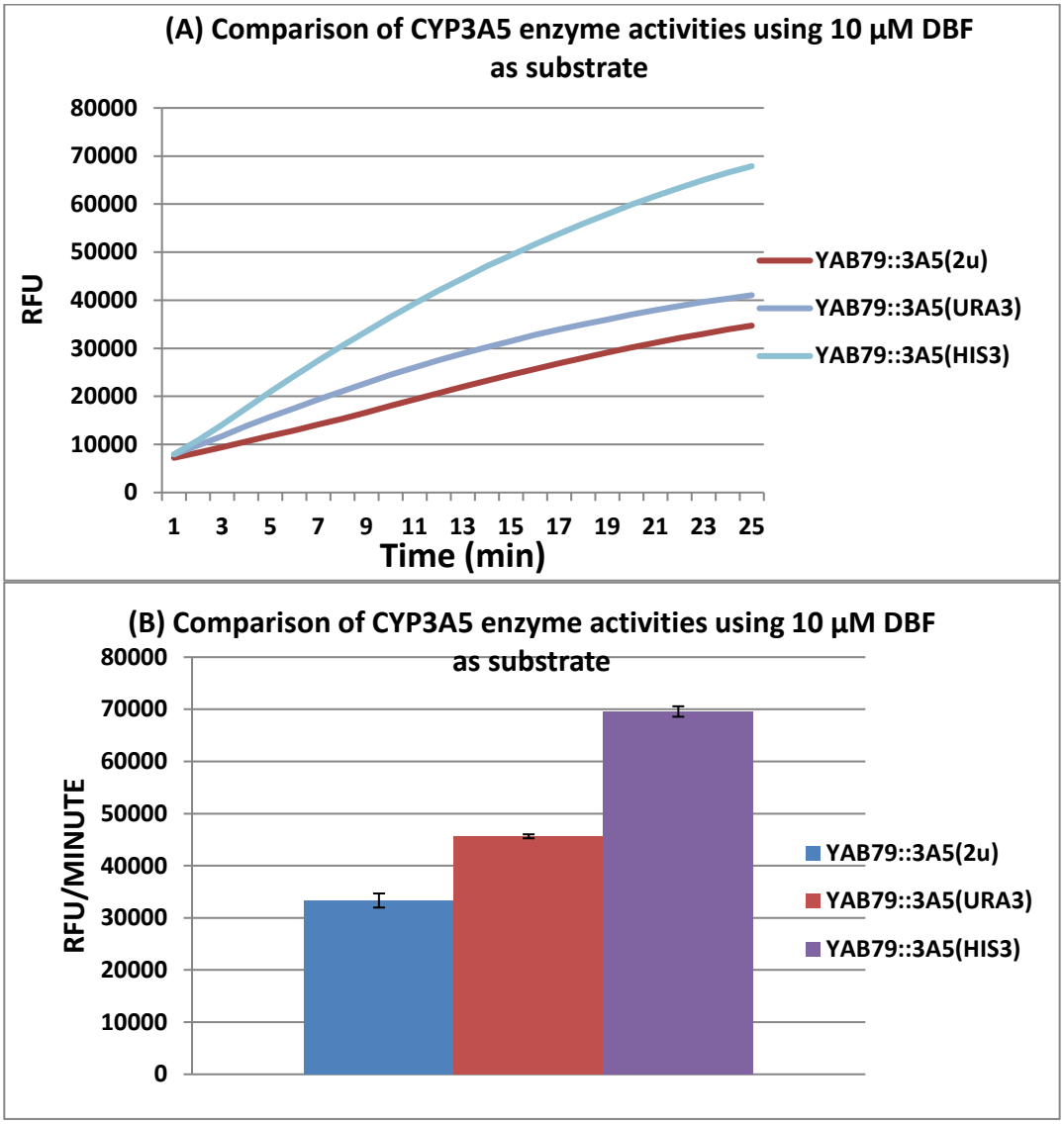


Figure 4.81. The graph (A) compares the CYP3A5 enzyme activities produced in the strains YAB79::3A5(HIS3), YAB79::3A5(URA3) with the strain YAB70::3A5(2µ) expressing CYP3A5 from an episomal, 2-micron (2u) plasmid. The graphs represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

Figure 4.81 shows that the *HIS3* locus provides the best expression of a single copy of the *CYP3A5_yc* gene in the yeast strain YAB79. It is better than when it is expressed from the *URA3* locus. The results also show that expression from a chromosomal locus is better than that from an episomal, 2-micron (2µ) plasmid. Further corroboration of these results is revealed in Chapter 5 of this study.

Figure 4.81 suggests that the amounts of CYP*3A5* expressed depend on the genetic locus and the chromosome in which the *CYP3A5_yc* gene expression cassette is integrated.

4.15 Construction of yeast strains, bearing expression cassettes of the human CYP2C8 gene, chemically synthesized using yeast biased codons, and comparison of CYP2C8 enzyme activities produced by different strains

4.15.1 Construction of a yeast integrative plasmid that would allow stable expression of human CYP2C8 enzyme from the HIS3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpHisADH2S/CYP2C8_yc was created via the following steps:

(1) After digestion of the plasmid YIpHisADH2S (Figure 4.11) with restriction enzymes *BamH*I, *Xba*I, the 5305 bp vector fragment was isolated.

(2) Isolation of a 1497 bp *BamH*I-*Xba*I *CYP2C8_yc* gene fragment. The gene was synthesized (Genewiz) using yeast-biased codons based on the protein template

with NCBI Accession Number, NM_000770. The gene was isolated from a pUC57 based plasmid into which the chemically synthesised gene had originally been cloned.

(3) The 5305 bp vector and the 1497 bp insert were ligated to create the plasmid YIpHisADH2S/CYP2C8_yc (Figure 4.82).

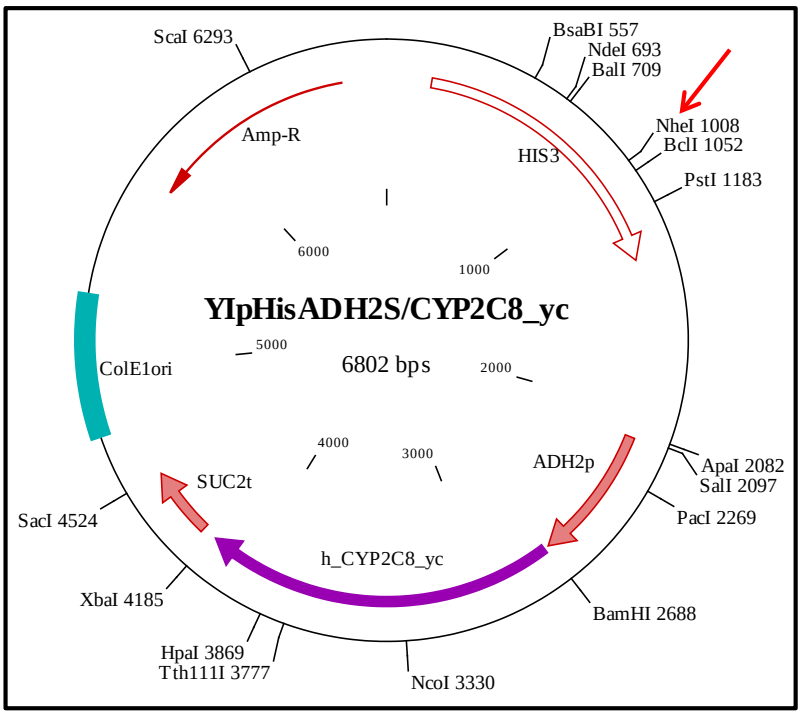


Figure 4.82. Map of plasmid YIpHisADH2S/CYP2C8_yc that allows integration of a human CYP2C8 gene expression cassette at the HIS3 locus of the yeast genome. The human CYP2C8 gene was synthesized using yeast-biased codons and was named h_CYP2C8_yc. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpHisADH2S/CYP2C8_yc was linearized at the *Nhe*I site (indicated by the arrow in Figure 4.82). In order to facilitate homologous recombination, the restriction sites *BsaB*I, *Nde*I, *Bal*I or *Bcl*I (Figure 4.82) could also have been used for linearization.

The newly constructed plasmid YIpHisADH2S/CYP2C8_yc was further analysed via digestion with *Pvu*II and *Xho*I restriction enzymes (Figure 4.83). The agarose gels show the expected sizes of DNA fragments.

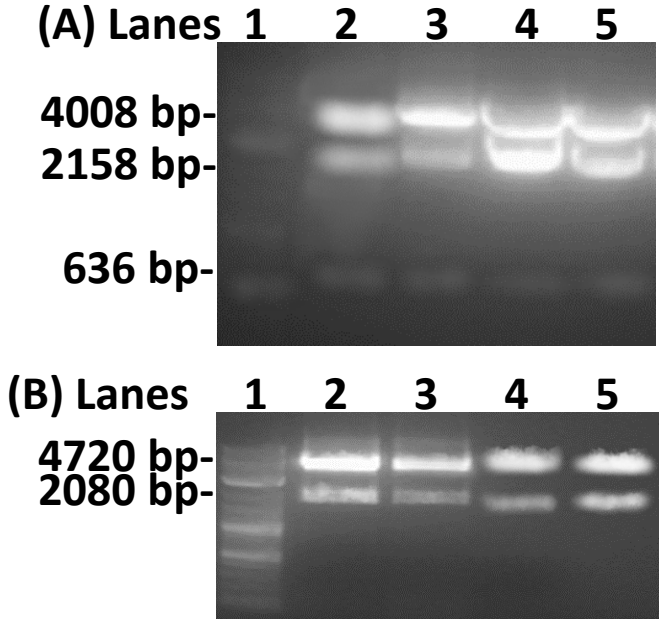


Figure 4.83. Agarose gels (A, B) that show the expected DNA fragments of YIpHisADH2S/CYP2C8_yc when plasmids isolated from four (A, B) bacterial clones were digested with PvuII (lanes 2-5; A) and XhoI (lanes 2-5; B). Lane 1 (A & B), 2-log DNA ladder.

The YIpHisADH2S/CYP2C8_yc plasmid can be used for expression of the *CYP2C8_yc* gene driven by the *ADH2* promoter. In order to allow this, the *CYP2C8_yc* gene expression cassette was integrated into the *HIS3* locus on chromosome XV.

4.15.2 Construction of a yeast strain that contains a copy of the CYP2C8_yc gene expression cassette integrated into HIS3 chromosomal loci of the yeast strain YAB79

A yeast strain, containing a single copy of the *CYP2C8_yc* gene expression cassette, was obtained by integrating the plasmid that bears the *CYP2C8_yc* expression cassette in the yeast strain YAB79 (BC300::ΔhRDM/LEU2$^+$, b5/TRP1; Figure 4.26) that expresses ΔhRDM from the *LEU2* locus and cytochrome b5 from the *TRP1* locus.

The *CYP2C8_yc* gene encoding plasmid that was used for integration in the YAB79 strain was YIpHisADH2S/CYP2C8_yc.

After integration, the strain was named YAB79::2C8_yc(HIS3).

The amount of enzyme expressed from this strain was then compared with that from the strains that bears an episomal plasmid. Fluorescence assays, using an appropriate fluorogenic substrate, were used for this comparison. The comparative kinetic analysis of CYP2C8 enzyme, co-expressed with cytochrome b5 and cytochrome P450 reductase ΔhRDM, was performed using DBF as a substrate. The intact yeast cells bearing the *CYP2C8_yc* gene integrated in the *HIS3* chromosomal locus, the episomal plasmid bearing the *CYP2C8_yc* gene and the empty plasmid containing control yeast strain were grown as described in Section 4.1.1. Comparative enzyme kinetic assays were performed, as described in Section 4.1.2, to measure formation of the product, fluoroscein. These procedures were carried out in three independent experiments.

4.15.3 Comparison of activities of CYP2C8 enzyme expressed from (a) the CYP2C8_yc gene chromosomally integrated at the HIS3 locus and (b) an episomal plasmid bearing the CYP2C8_yc gene, in the yeast strain YAB79

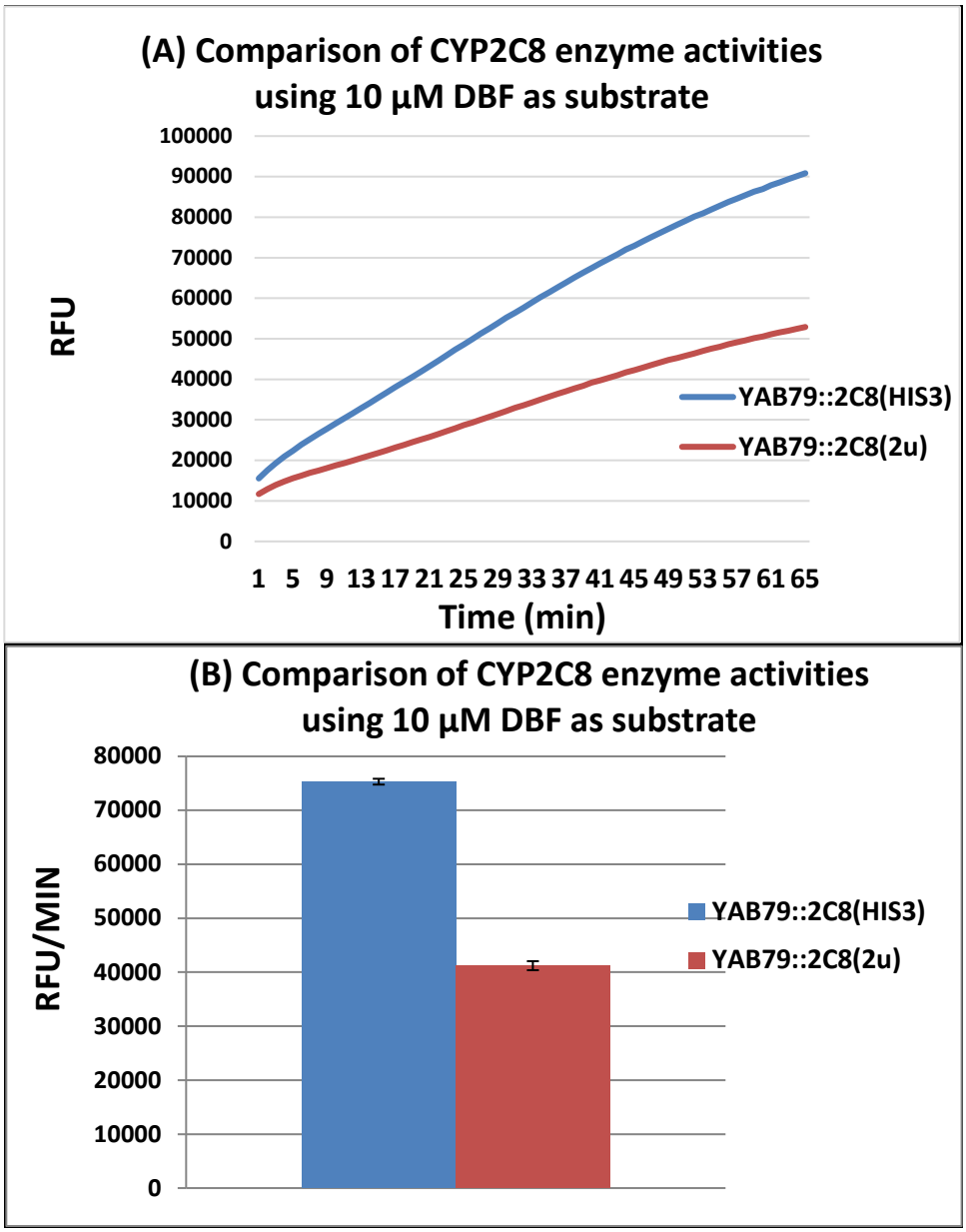


Figure 4.84. The graph (A) compares the CYP2C8 enzyme activities produced in the strain YAB79::2C8(HIS3) with the strain YAB70::2C8(2µ) that expresses CYP2C8 from an episomal, 2-micron (2u) plasmid. The graphs represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

Figure 4.84 shows that the expression levels of human CYP2C8 enzyme are better when expressed from a chromosomal locus than when it is expressed from an episomal, 2-micron (2μ) plasmid.

4.16 Construction of yeast strains, bearing expression cassettes of the human CYP2E1 gene, chemically synthesized using yeast biased codons, and comparison of CYP2E1 enzyme activities produced by different strains

4.16.1 Construction of a yeast integrative plasmid that would allow stable expression of human CYP2E1 enzyme from the HIS3 chromosomal locus of a yeast strain

The yeast integrative plasmid YIpHisADH2S/CYP2E1 _yc was created via the following steps:

(1) After digestion of the plasmid YIpHisADH2S (Figure 4.11) with restriction enzymes *BamH*I, *Xba*I, the 5305 bp vector fragment was isolated.

(2) Isolation of a 1506 bp *BamH*I-*Xba*I *CYP2E1_yc* gene fragment. The gene was synthesized (Genewiz) using yeast-biased codons based on the protein template with NCBI Accession Number, NM_000773. The gene was isolated from a pUC57 based plasmid into which the chemically synthesised gene had originally been cloned.

(3) The 5305 bp vector and the 1506 bp insert were ligated to create the plasmid YIpHisADH2S/CYP2E1_yc (Figure 4.85).

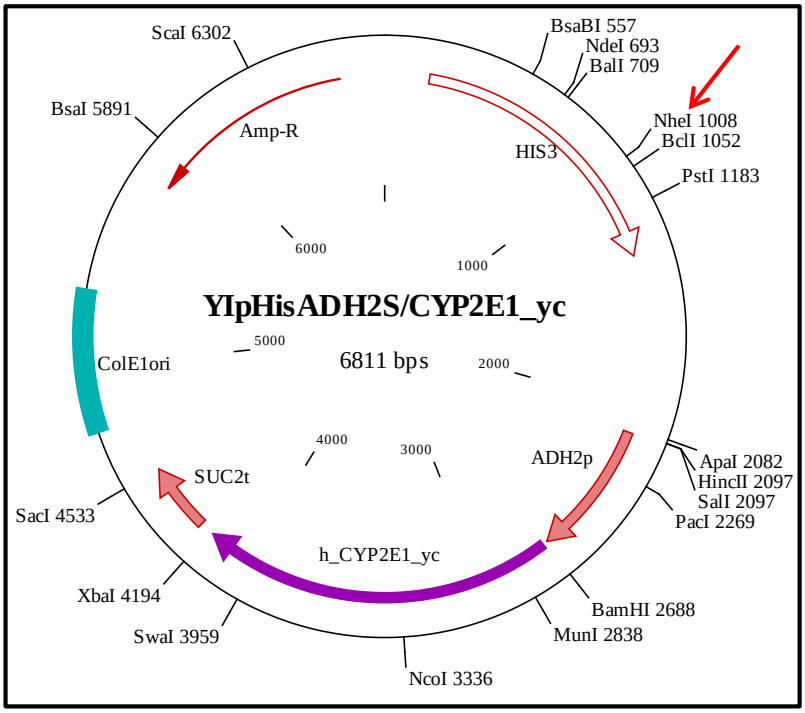


Figure 4.85. Map of plasmid YIpHisADH2S/CYP2E1_yc that allows integration of a human CYP2E1 gene expression cassette at the HIS3 locus of the yeast genome. The human CYP2E1 gene was synthesized using yeast-biased codons and was named h_CYP2E1_yc. The map shows restriction sites that cut the plasmid only once.

For integration into yeast cells, via homologous recombination, the plasmid YIpHisADH2S/CYP2E1_yc was linearized at the *Nhe*I site (indicated by the arrow in Figure 4.82). In order to facilitate homologous recombination, the restriction sites *BsaB*I, *Nde*I, *Bal*I or *Bcl*I (Figure 4.85) could also have been used for linearization.

The newly constructed plasmid YIpHisADH2S/CYP2E1_yc was further analysed via digestion with the restriction enzyme *Xho*I (Figure 4.86). The agarose gel shows the expected sizes of DNA fragments.

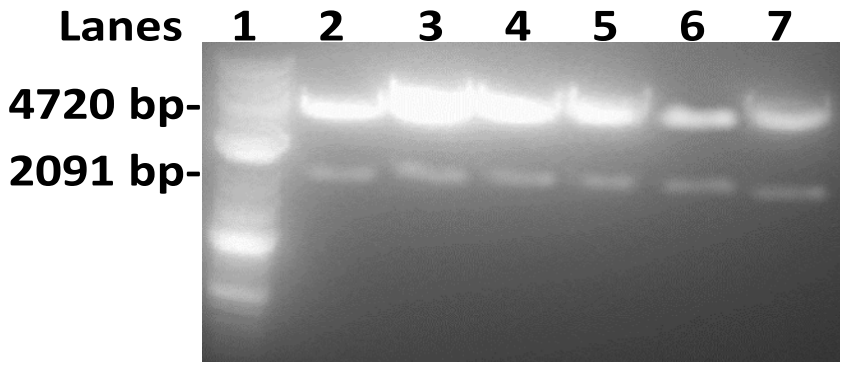


Figure 4.86. Agarose gel that shows the expected DNA fragments of YIpHisADH2S/CYP2E1_yc when plasmids isolated from six bacterial clones were digested with XhoI (lanes 2-7). Lane 1, 2-log DNA ladder.

The YIpHisADH2S/CYP2E1_yc plasmid can be used for expression of the *CYP2E1_yc* gene driven by the *ADH2* promoter. In order to allow this, the *CYP2E1_yc* gene expression cassette was integrated into the *HIS3* locus on chromosome XV.

4.16.2 Construction of a yeast strain that contains a copy of the CYP2E1_yc gene expression cassette integrated into HIS3 chromosomal loci of the yeast strain YAB79

A yeast strain, containing a single copy of the *CYP2E1_yc* gene expression cassette, was obtained by integrating the plasmid that bears the *CYP2E1_yc* expression cassette in the yeast strain YAB79 (BC300::ΔhRDM/LEU2$^+$, b5/TRP1; Figure 4.26) that expresses ΔhRDM from the *LEU2* locus and cytochrome b5 from the *TRP1* locus.

The *CYP2E1_yc* gene encoding plasmid that was used for integration in the YAB79 strain was YIpHisADH2S/CYP2E1_yc.

After integration, the strain was named YAB79::2E1_yc(HIS3).

The amount of enzyme expressed from this strain was then compared with that from the strains that bears an episomal plasmid. Fluorescence assays, using an appropriate fluorogenic substrate, were used for this comparison. The comparative kinetic analysis of CYP2E1 enzyme co-expressed with cytochrome b5 and cytochrome P450 reductase ΔhRDM, was performed using EOMCC as a substrate. The intact yeast cells bearing the *CYP2E1_yc* gene integrated in the *HIS3* chromosomal locus, the episomal plasmid bearing the *CYP2E1_yc* gene and the empty plasmid containing control yeast strain were grown as described in Section 4.1.1. Comparative enzyme kinetic assays were performed as described in Section 4.1.2 to measure formation of product, 7-HCC. These procedures were carried out in three independent experiments

4.16.3 Comparison of activities of CYP2E1 enzyme expressed from (a) the CYP2E1_yc gene chromosomally integrated at the HIS3 locus and (b) an episomal plasmid bearing the CYP2E1_yc gene, in the yeast strain YAB79

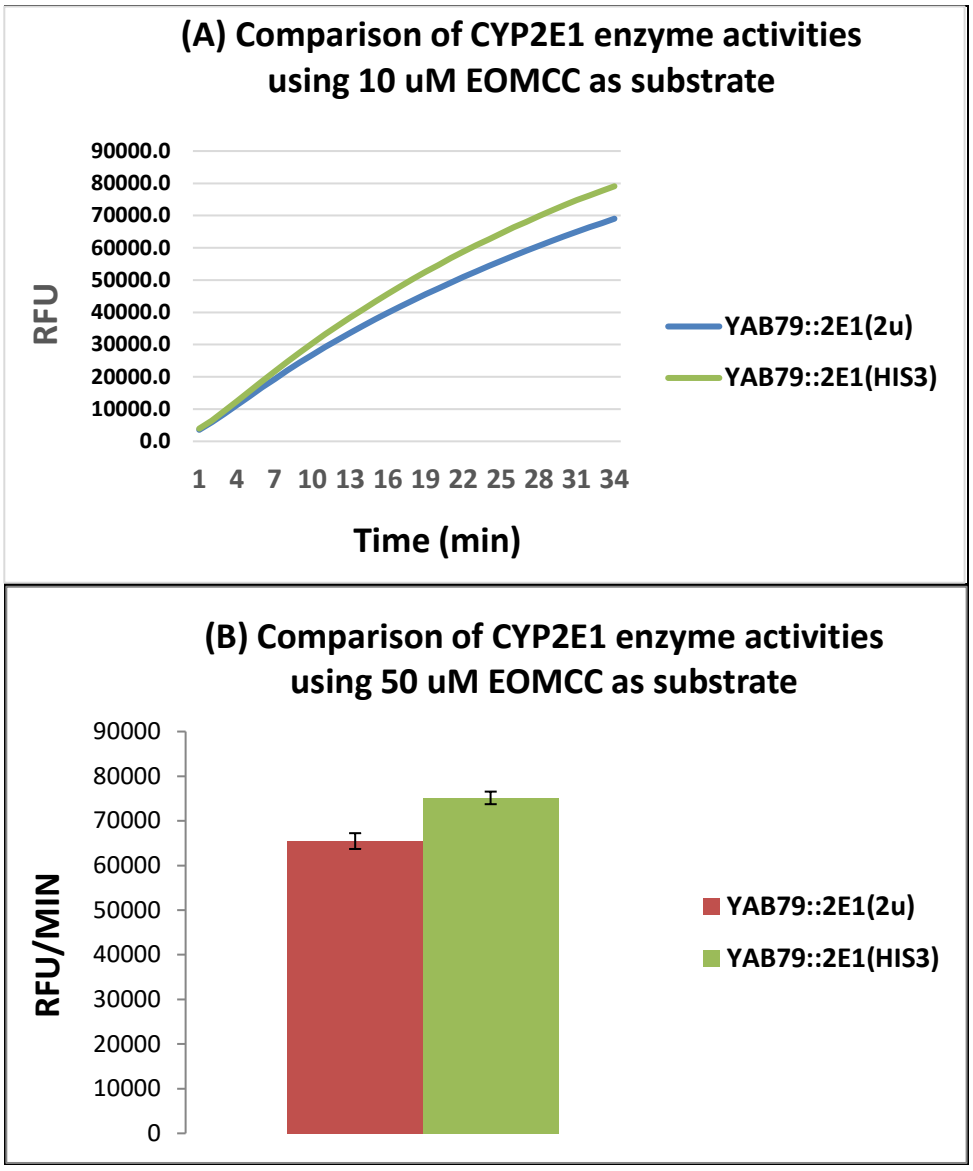


Figure 4.87. The graph (A) compares the CYP2E1 enzyme activities produced in the strain YAB79::2E1(HIS3) with the strain YAB70::2E1(2µ) that expresses CYP2E1 from an episomal, 2-micron (2u) plasmid. The graphs represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

Figure 4.87 suggests that the expression level of human CYP2E1 enzyme is slightly better when expressed from an integrated locus than expression from an episomal, 2-micron (2μ) plasmid.

4.17 Conclusion

In this Chapter (i.e. Chapter 4), I have cloned and expressed genes that code for the CYPs 1A2, 1A1, 1B1, 3A4, 2C9, 2C19, 4F3A, 3A4, 2E1, 2C8, 2D6(1), and 2D6(2). Integration into chromosomal loci has shown greater enzyme activity than from an episomal plasmid. This may correlate with (a) higher expression levels and (b) higher activity of the enzymes for all the CYPs that have been expressed. This has been tested further in Chapter 5 where results corroborate that both assumptions are probably correct.

The insect cell-baculovirus expression system is extensively used for the expression of recombinant proteins. The two leading manufacturers of recombinant human CYP enzymes produce them using the baculovirus expression system. In general, a baculovirus which has a stably integrated copy of a foreign gene is used to infect insect cells. However, in this system of expression, production of recombinant proteins is generally low compared to that obtained in bacterial cells (Rosano and Ceccarelli, 2014). Nonetheless, the human CYP enzymes produced in this system are considered more authentic (i.e. more akin to enzymes produced in human cells) than that produced form bacterial cells since insect cells are eukaryotic.

Yeast is also a eukaryotic organism. It can provide comparable levels of expression of foreign proteins as bacterial cells. The advantage of expressing human CYPs in eukaryotic yeast is that it possesses ER membranes. Human CYPs, which all contain an

N-terminal membrane-binding domain, are naturally bound to the ER membranes. Yeast can provide a natural internal environment in which human CYP enzymes could be bound to its ER membranes. Binding to the ER provides active CYP proteins. Soluble CYPs are totally inactive. However, until now, expression of human CYPs in yeast has not been a great success. Usually, expression has been seen at low levels (Zimmermann et al.,2011).

In Chapter 3, we had found that synthesising the human CYP genes with yeast biased codons provides higher levels of CYPs compared to the native genes. In this Chapter we find that integrating a *CYP* gene, synthesized with yeast biased codons, at a particular chromosomal locus yields higher levels of CYPs than expression from the same gene borne on an extra-chromosomal plasmid. The use of the inducible *ADH2* promoter further helps in the expression of human CYP proteins. Usually, inducible promoters switch on gene transcription very quickly in the presence of the inducer. In the case of the inducible yeast *GAL* (*GAL1*/*10*) promoters, galactose is the inducer whereas for the yeast metallothionein promoter, *CUP1*, it is the divalent Cu^{2+} cation. During the use of the *GAL1*/*10* and the *CUP1* promoters, the cells have to quickly adapt to the production of foreign proteins, after the inducer has been added, which could be very stressful for the cells. During production of foreign proteins with the *ADH2* promoter, cells however can slowly adapt to the protein that is being produced. As glucose is being utilized by yeast cells for growth, the ethanol that is made gradually induces the promoter, thereby allowing time for the cells to gently adjust to the foreign protein that is being expressed.

We believe that this unique conglomeration of features facilitates high yields of CYPs that have been obtained until now, in this thesis. Integration of *CYP* genes on to the chromosome also allows yeast cells to be grown in complete medium to obtain large

volumes of cells compared to cells grown in synthetic SD minimal medium; cells containing an episomal plasmid must be grown in SD medium for maintenance of the plasmid.

Using the technology for targeting genes into chromosomal loci, the importance of CBR (cytochrome b5 reductase) was clarified to some extent. CYP2D6 expression level was compared when co-expressed with and without CBR's substrate cytochrome b5. Our results show that for both the CYP2D6(1) and CYP2D6(2) variants, CBR does not aid production of these CYP variants; in fact, it is deleterious (Figure 4.45). In contrast, CBR has a role to play in the expression of CYP1A2 since cytochrome b5 definitely augments CYP1A2 expression levels (i.e. activity) (Figure 4.37). This has never been reported before (Duarte et al., 2005). Besides, it was clearly seen that cytochrome b5 has an effect on the levels/activities of CYP3A4, CYP3A5, CYP2C8, CYP2C9, CYP2C19, CYP2E1, and CYP4F31A enzymes. CYP3A4 is involved in the metabolism, in the liver, of more than 50% of all approved drugs.

The CYP3A4 and CYP3A5 enzymes isolated from insect cells using baculoviruses (Corning-Gentest's Supersomes) have been used to test which of these enzymes metabolise vinorelbine, an alkaloid used in the treatment of breast and non-small lung cancers. It was concluded that vinorebine clearance is closely associated with CYP3A4 activities but not with that of CYP3A5 (Ariel et al., 2013). It would be reasonable to assume that due to the high levels of human CYP enzyme expression in yeast, it will be of great advantage to use these CYP enzymes expressed within yeast for routine drug metabolism studies. The recombinant yeast cells would also be robust and cost effective means of conducting such studies.

Expression of integrated copies of *CYP1A1* and *CYP1B1* genes have shown that the levels of CYP enzymes produced is much higher when compared to expression from strains containing episomal plasmids that encode these genes. Whole yeast cells, expressing these two enzymes, have been used for the biotransformation of 5,7-dihydroxyflavone (chrysin) to 5,6,7-trihydroxyflavone (baicalein) via regio-selective C6-hydroxylation with greater than 90% yield (see Chapter 6, Section 6.3; Ibidapo WIlliams et al., 2017, published online; in press). *Baicalein is known for its fascinating anticancer properties in various cancer types including pancreatic, gastric, colorectal cancer,* multiple myeloma, head and neck, and breast cancers (Roy at al., 2007).

Cells expressing CYP2C9 and CYP3A4 have also been used, during the course of the present study, on nano-electrodes to develop biosensors for the two anticoagulants warfarin and rivaroxaban (yet unpublished observations). The approach is expected to be useful in diagnostics. Our results indicate that all major clinically important CYP450 enzymes could be integrated as potential biosensors to accurately test the levels of medicines (as CYP450 substrates) in patients' blood. This platform can be developed further to create point-of care or at home monitoring devices to detect all CYP enzyme-metabolised medicines. This would provide useful information on drug dosages which patients themselves can adjust if and when required. .

In conclusion, the results presented in this Chapter are likely to open up new avenues towards further research in the use of whole yeast cells which highly express human CYP450 enzymes.

Chapter 5 Isolation of Sacchrosomes (i.e. CYP enzymes bound to yeast microsomal membranes), from yeast strains expressing two integrated copies of a CYP, and comparison of their activities with commercially available microsomal enzymes isolated from insect and bacterial cells

5.1 Introduction

Chapter 4 described the development of yeast strains which contained one chromosomally integrated copy of a human *CYP* gene expression cassette. These strains allowed expression from the yeast cells' different chromosomal locations at which the *ADE2*, *HIS3* or *URA3* genes were situated. It was observed that a strain containing an integrated copy of a single *CYP* gene unexpectedly produces much higher levels of the CYP protein than a strain containing an episomal, 2-micron (2µ) plasmid which ought to produce multiple copies of the gene product (Falcon et al., 2005). After a pre-culture in a selective medium, which selects for the *URA3* gene present in both episomal and integrative plasmids, the two strains were grown in non-selective full medium for 18 h (i.e. a time period in which an episomal plasmid undergoes minimal plasmid loss). Fluorogenic (i.e. non-fluorescent) substrates which, upon reaction with CYP enzymes, produce fluorescent products were used for these comparative studies. It was inferred that greater the amount of fluorescence (i.e. the fluorescent product) detected from the cells of a specific strain, higher the level of CYP enzyme that is produced within these cells.

Integration of a *CYP* gene expression cassette in a chromosomal locus overcomes the problem of instability (i.e. plasmid loss) when cells are grown in full YPD medium which

is normally encountered by cells harbouring an episomal plasmid. Although a highly nutritious full medium allows healthy growth of cells, they are unable to enforce the continuous presence of an extra-chromosomal plasmid (i.e. an episomal plasmid) within the cells during cell division. Gradual loss of an episomal plasmid starts at around ten generations of growth in YPD, each generation lasting ~90 min (Lopez et al, 1996); a 'generation' in yeast's cell division is defined as the time taken to double the number of cells.

A gradual loss of an episomal plasmid bearing a heterologous human gene (i.e. in this case, the human *CYP* gene), in YPD medium, would lead to steady decline in protein production and, hence, decrease in CYP activity. Complete loss of CYP activity can be seen within 3-5 days of growth. However, once integrated at a chromosomal locus, a *CYP* gene expression cassette can never be lost when grown in YPD medium. Only when yeast cells become extremely stressed, they may undergo apoptosis, which is a form of programmed cell death. Apoptosis could occur because (a) the conditions of cell culture produce a toxic chemical entity and/ or (b) the cells produce large amounts of protein, which is inherently toxic to the cell.

The consequence of growth of yeast cells in YPD medium is in stark contrast to growth in selective minimal SD medium. Growth of cells in SD medium prevents plasmid loss by selecting for the presence of an extra-chromosomal plasmid through its auxotrophic marker, as long as essential nutrients within the medium are present. This implies that yeast cells that lack the *URA3* gene, but contain an episomal plasmid (such as pSYE263; Figure 5.1) which bears the *URA3* gene besides encoding an expression cassette for a heterologous *CYP* gene or any other gene of choice, can be selected in SD minimal

medium lacking uracil. This implies that only those yeast cells that contain the *URA3* bearing episomal plasmid will grow on such medium. Unfortunately, however, SD medium, which is deprived of most of the essential nutrients present in YPD medium, allows only miniscule levels of cell growth compared to growth of cells in YPD. Less than 5% of cell growth is seen in SD medium when compared to growth of yeast cells in YPD.

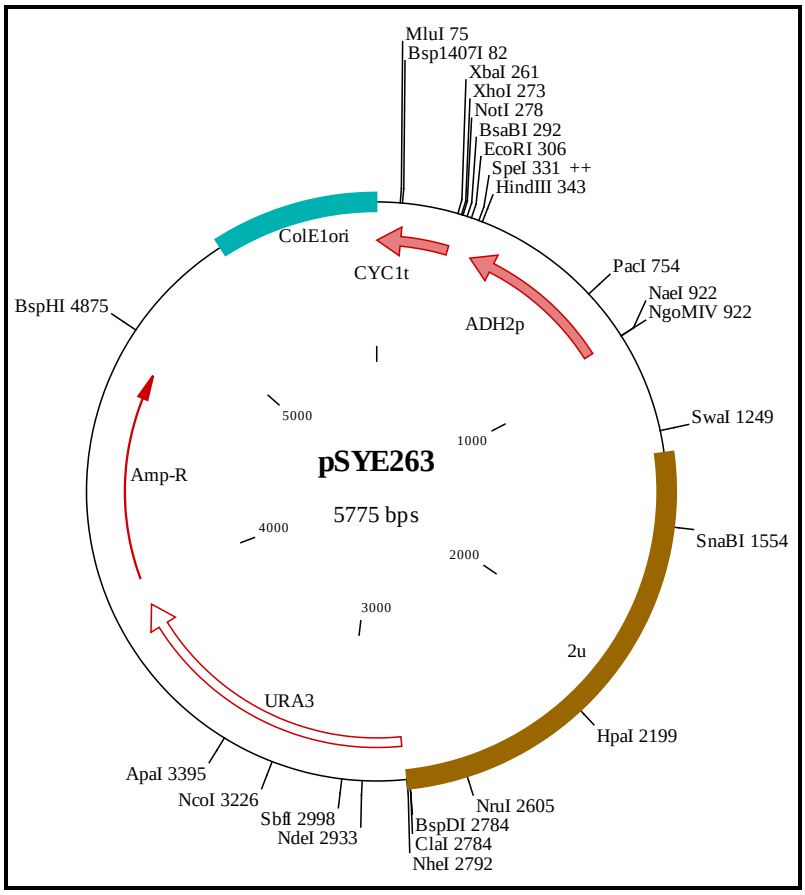


Figure 5.1. An episomal, 2-micron (2u) based plasmid, pSYE263. It contains the functional URA3 gene that allows selection of the plasmid in yeast cells that contain a mutant, dysfunctional ura3 gene, when cells are grown in minimal SD medium that lacks uracil. The restriction sites that occur only once in the plasmid are shown.

The complete stability of yeast strains that contain an integrated copy of a *CYP* gene expression cassette can provide an exciting and beneficial avenue towards industrial scale use of these strains. In this Chapter, attempts have been made to improve on the levels of CYP proteins that were already obtained from such integrated strains. It was logical to think that a second copy of a *CYP* gene expression cassette would further improve CYP levels obtained in yeast cells than what was already observed in the one-copy strains, described in Chapter 4. However, there were also serious doubts about the feasibility of introduction of a second copy. It could be that overexpression of CYPs could lead to toxicity whereby cells would have to adapt spontaneously to produce decreased levels of CYPs to avoid toxic levels of the protein. CYPs are redox proteins, overproduction of which could be potentially toxic. Side by side, overproduction of a potentially toxic protein could lead to the possibility of seeing less cell growth.

To explore further, it was decided to introduce a second copy of a *CYP* gene to the existing one-copy *CYP* gene containing yeast strains. Jensen et al (2014) have integrated, via homologous recombination, three genes coding for different fluorescent proteins. This was attempted to show that multiple genes can be integrated into a yeast strain and be functionally expressed. All three gene expression cassettes, under the control of the constitutive *GAPDH* promoter, were integrated on the same chromosome (i.e. chromosome X) of the genome of a yeast *S. cerevisiae* strain. Fluorescence at different emission/absorption wavelength was monitored to show only that the three proteins were merely expressed. It was seen that expression occurred at very low levels.

As was plainly seen in Chapter 4, there were different levels of CYP enzyme expression from the *HIS3* and the *URA3* chromosomal loci of yeast cells. Integration of a *CYP* gene

expression cassette at the *HIS3* chromosomal locus produces more CYP proteins than from the *URA3* locus. *HIS3* belongs to yeast chromosome XV and the *URA3* gene is an integral part of chromosome V. It can be speculated that, during expression with an inducible promoter (i.e. ADH2p), proteins neighbouring the *HIS3* and *URA3* genes in chromosomes XV and V, where the heterologous *CYP* genes are embedded, may have a differential effect on *CYP* gene transcription, mediated by the ADH2p. These adjacent proteins, when in *trans*, may be acting differentially to produce different amounts of a CYP enzyme. In order to avoid the possibility of these proteins, which act in *trans* to enhance gene expression, being limiting it was decided to integrate two copies of a *CYP* gene expression cassette at two separate chromosomal loci, that is, at the *HIS3* and the *URA3* loci of yeast cells. This would allow stable expression of a *CYP* gene of choice, without any loss of plasmid in full YPD medium and would avoid the limitations of factors that may be aiding gene transcription.

Metabolic engineering of the yeast *Saccharomyces cerevisia*e, with the aim of producing important chemicals relevant for the sustenance of human beings, has made huge progress in recent years. This has paved the way for advancing the genetic tools needed for the expression of heterologous proteins in yeast (Krivoruchko et al., 2011). Work in this Chapter has used methods related to chromosomal integration, which allows permanent attachment of a heterologous gene of interest at a chromosomal locus so that the corresponding protein could be expressed consistently in full YPD medium without loss of the gene from the cells. As described earlier, in the absence of selection pressure in YPD medium, which allows vigorous growth, an episomal plasmid is lost from the cells. An episomal plasmid can be maintained only in minimal SD medium, which, in contrast to YPD medium, unfortunately permits only poor growth of cells.

Most importantly, the microorganism yeast *S. cerevisiae* provides a robust system that possesses highly active machinery for homologous recombination. This yeast contains a conglomeration of enzymes that constitutes a platform for reliable integration of linearized DNA, which contains specific gene expression cassettes, into the yeast genome. The linearized DNA is flanked by DNA pieces which are homologous (i.e. identical) to regions in the yeast chromosome that encode auxotrophic markers. Mutations within genes encoding enzymes in biosynthetic pathways that lead to the synthesis of the basic metabolic building blocks, such as an amino acid or a nucleotide, are referred to as auxotrophic markers. They are used as selection markers during the process of transformation of an episomal plasmid or a linearized DNA. Transformation of an episomal plasmid leads to extra-chromosomal propagation of the plasmid whereas the linearized DNA through transformation (i.e. the process of introduction into cells) becomes permanently embedded on a particular site, where an auxotrophic marker lies, on the chromosome.

Various methods for integration of a linearized plasmid have been described in the literature. They include 'DNA assembly' (Shao and Zhao, 2009) and 'reiterative recombination' (Wingler and Cornish, 2011). These methods were adapted to create a protocol that facilitates integration of linearized pieces of DNA, encoding different gene expression cassettes, into a single locus on a particular chromosome of the yeast genome. In order to circumvent the problem of genes on a single chromosome influencing the expression of each other at the transcriptional level (as mentioned above), it was decided that we introduce the second and, in some cases, the third copy of the *CYP* genes into different chromosomal locations. This would allow later on (as in Chapters 6 and 7), independent of transcriptional interference, investigations on the effects of the expression

of more than one CYP protein on each CYP's active site geometry. This is often referred to as 'neighbouring effect' or 'crowding effect' that occurs through the simultaneous expression of proteins belonging to the same family (Bryant et al., 2014).

In summary, in this Chapter (i.e. Chapter 5), the hypothesis that integration of 2 or 3 copies of *CYP* genes on different chromosomal loci will provide higher levels of protein, visualized through monitoring of their activities via fluorescence assays, than one integrated copy has been explored by performing experiments with different *CYP* genes.

5.2 Outline of this Chapter

This Chapter is a continuation of Chapter 4. Here, two (in one case, three) copies of human *CYP* genes (chemically synthesized with yeast biased codons) have been integrated at two (or three) different chromosomal loci, via homologous recombination. The aim was to express human CYP enzymes within baker's yeast cells, at high levels and with enhanced activity. The ultimate goal was to create robust recombinant yeast strains that would allow isolation of microsomal [i.e. endoplasmic reticular (ER) membrane bound] enzymes from these cells, in high yields and with activities which were better than what has been published before. CYPs are totally inactive when shorn off the microsomal (ER) membranes. To be able to isolate these fragile microsomal enzymes in enhanced yields and activities, a new process was developed.

In order to achieve the goal, a number of yeast strains were created and they were individually screened for CYP enzyme activity, via fluorescence assays using fluorogenic substrates. The enzyme activities for a particular CYP, from different clones of the same strain, were then compared. It was inferred that enzyme activities correlate with the

amounts of CYPs within the cells. This was verified by isolating microsomal human CYP enzymes (i.e. bound to ER membranes) from the best and worst CYP producing clones that were identified through the initial screening process. The activities of baker's yeast derived microsomal enzymes (Sacchrosomes) were then compared, head-to-head, with the three commercially available enzymes, which are, produced either from insect or bacterial cells and which are sold worldwide. Global market size of recombinant human CYPs is considered to be at least $250 million. The results obtained show that the human CYP enzymes, produced from baker's yeast, are 2-3 times more active than the commercially available enzymes. The results also suggest that, without embarking on production of these enzymes in a cheaper environment, the human CYP enzymes could easily be produced in the UK and sold at half the price quoted by the current manufacturers. Yet these cheap, highly active enzymes, if marketed, could still provide a profitable margin. Because of their cheapness, they could be made widely available for early pre-clinical research which is not possible right now because recombinant human CYP enzymes that are commercially available are so expensive (i.e. £150-200 per nanomole).

5.3 Results

5.3.1 Fluorescence-based assays for determining CYP enzyme activities in whole cells and isolated microsomes

Only a few chemical molecules have been found to be useful as selective probes (i.e. as inhibitors and substrates) of each human CYP enzyme. Analysis of CYP enzyme inhibition and identification of metabolites that are formed, upon reaction of a CYP enzyme with its substrate, involves the use of techniques such as high-pressure liquid chromatography (HPLC) and liquid chromatography in tandem with mass spectrometry (LC-MS). These methodologies are slow and massively labour intensive. Alternatively, for rapid screening of libraries of chemical compounds for their potential to inhibit CYP enzymes, fluorogenic substrates are used. These substrates do not fluoresce on their own but form fluorescent products upon reacting with specific CYP enzymes.

Several substrates have been used to analyse metabolite formation for different CYP enzymes. For example, non-fluorescent or low-fluorescent P450 substrates have been used that produce highly fluorescent metabolites in aqueous solutions. Historically, the very early substrates had limited aqueous solubility and poor metabolite formation; it gave a high background and low signal to noise ratio, which was not appropriate for screening of CYP enzyme inhibitors. Later on, *O*-alkyl derivatives of resorufin, fluorescein, 7-hydroxycoumarins and other molecules were discovered to study CYP-mediated fluorescent metabolite formation (Burke at al., 1985 & Stresser et al., 2000).

Some non-fluorescent probes (substrates) are selective and have been reported to be useful for measuring individual CYP enzyme activities (Chauret at al., 1999, 2001).

However, it was also realised that non-selective substrates would not be good enough for measuring CYP450 activities in human liver microsomes in vitro because there are 12-15 of the CYP enzymes which are co-expressed in the human liver. However, this limitation of the substrates being non-selective can be overlooked in the case of recombinant membrane-bound CYP enzymes, which are individually expressed within cell factories. Hence, for recombinant microsomal CYP enzymes, non-selective substrates can be used for testing individual CYP activities.

In this study, a battery of fluorescence-based assays have been described for rapid measurement of different CYP enzyme activities expressed within intact yeast cells and in microsomes isolated from these cells which express individual CYP enzymes. The cultured yeast cells were washed with 1X TE buffer, as was described in Materials and Methods (Chapter 2) before directly incubating them with a fluorogenic substrate (i.e. a non-fluorescent substrate which can produce a fluorescent product upon reaction with a CYP enzyme). The metabolites (i.e. products) formed were quantified fluorometrically by measuring the fluorescence emitted by the product, on a plate reader.

These assays also:

(a) gave the possibility of clonally selecting sub-sets of cells that express high CYP activities and also

(b) provided the chance of analysing the inhibitory effects of new chemical compounds individually on each CYP enzyme.

The standard curves of the products formed by reaction of substrate with individual microsomal enzymes were obtained. The activities of isolated CYP microsomes were

then measured using fluorescence substrates, after incubation of enzyme with substrate for a few minutes. It was found the results obtained from intact cells and isolated microsomes were similar.

In this study some of the non-fluorescent (fluorogenic) substrates, listed below (Table 5.1), have been used to establish the amount of fluorescent metabolites that were formed during CYP-mediated metabolism of substrates.

Table 5.1. The non-fluorescent substrates and the fluorescent products formed in CYP enzyme assays.

CYP	Substrate	Metabolite Formed
1A2	CEC (3-cyano-7-ethoxycoumarin)	CHC (3-cyano-7-hydroxycoumarin)
2A6	Coumarin	Hydroxycoumarin
2B6	EFC (7-ethoxy-trifluoromethylcoumarin)	HFC (7-hydroxytrifluoromethylcoumarin)
2C8	DBF (Dibenzylfluorescein)	Fluoroscein
2C9	MFC (7-methoxy-trifluoromethylcoumarin)	HFC (7-hydroxytrifluoromethylcoumarin)
2C19	CEC (3-cyano-7-ethoxycoumarin)	CHC (3-cyano-7-hydroxycoumarin)
2D6	AMMC (3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin)	AHMC (3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-hydroxy-4-methylcoumarin**)**
2E1	MFC (7-methoxy-trifluoromethylcoumarin)	HFC (7-hydroxytrifluoromethylcoumarin)
3A	DBF (dibenzylfluorescein), BFC (7-benzoyloxy-4-trifluoromethyl coumarin) or BOMCC (3-cyano-7-(benzyloxymethoxy)-coumarin)	Fluoroscein or HFC (7-hydroxytrifluoromethylcoumarin)

5.3.2 Relative stability of a yeast strain containing an integrated copy of CYP1A2 gene compared to a strain that bears an episomal plasmid that encodes CYP1A2 gene

Before embarking on the creation of yeast strains that contain 2 copies of a *CYP* gene, the stability of prototypic strain that contains only a copy of a specific *CYP* gene was tested. To the strain YAB79, containing a modified human P450 reductase [ΔhRDM (Chapter 4) and cytochrome *b5*, was introduced one copy of the *CYP1A2* gene by integration in YAB79's *HIS3* chromosomal locus. The resultant strain named YAB79::1A2(HIS3) was used for this experiment. The cells pertaining to the strain YAB79::1A2(HIS3) were subjected to a long period of growth in which cells were repeatedly inoculated into fresh YPD full medium, after every 24 h of growth. YPD medium contains 2% glucose as carbon source for growth of cells. After every 24 h, the cell culture was spun down and fresh medium was added.

Glucose is known to affect a variety of processes in yeast *S. cerevisiae*. This includes induction of genes needed for glucose transport, protein synthesis and repression of genes required for cell growth on carbon sources other than glucose (Carlson, M. 1999). Another important role of glucose is in the transcription of certain yeast genes, although the mechanism via which glucose regulates gene expression has not been fully understood. In 1997, Joseph et al demonstrated that the genes that are regulated by the change from fermentative growth on glucose to oxidative growth pertain to a diauxic shift. Yeast cells enter diauxic shift when glucose becomes limiting. It involves switching cells' metabolism from glycolysis to aerobic utilization of ethanol and is characterized by decreased growth rate. This would mean that if ethanol was used as a carbon source, it

would result in the repression of certain genes involved in yeast cell growth. Joseph et al's study portrayed the findings via cell growth curves. It showed that, when glucose is exhausted, there is a shift in the expression of genes. Besides this, the authors observed that when yeast cells became stressed the metabolic activity of the cells was depressed. This was monitored via expression of the green fluorescent protein (GFP) during yeast cell growth. The fluorescence produced in stressed cells was inversely proportional to the fluorescence emanated by cells that were not stressed.

With the above information at hand, YAB79 cells were transformed (i.e. a process of introduction of DNA into live yeast cells) with an

(a) Episomal (pSYE263/h_CYP1A2_yc; Figure 5.2) and

(b) Integrating plasmid (YIpHisADH2S/CYP1A2_yc; Figure 5.3).

The resultant strains obtained after transformation were named

(i) YAB79::1A2(2µ), and

(ii) YAB79::1A2(HIS3).

The episomal plasmid allows extra-chromosomal replication of plasmid during cell division, whereas the integrating plasmid targets the plasmid to a yeast chromosome.

The two yeast strains YAB79::1A2(2µ) and YAB79::1A2(HIS3) were grown in YPD medium and their growth was monitored over 4 days. The cultures were started with an initial inoculum of equal number of cells. Both cell cultures were grown in exactly the same way, with fresh YPD medium being provided at identical time points of growth. The results of growth are shown in Figure 5.4.

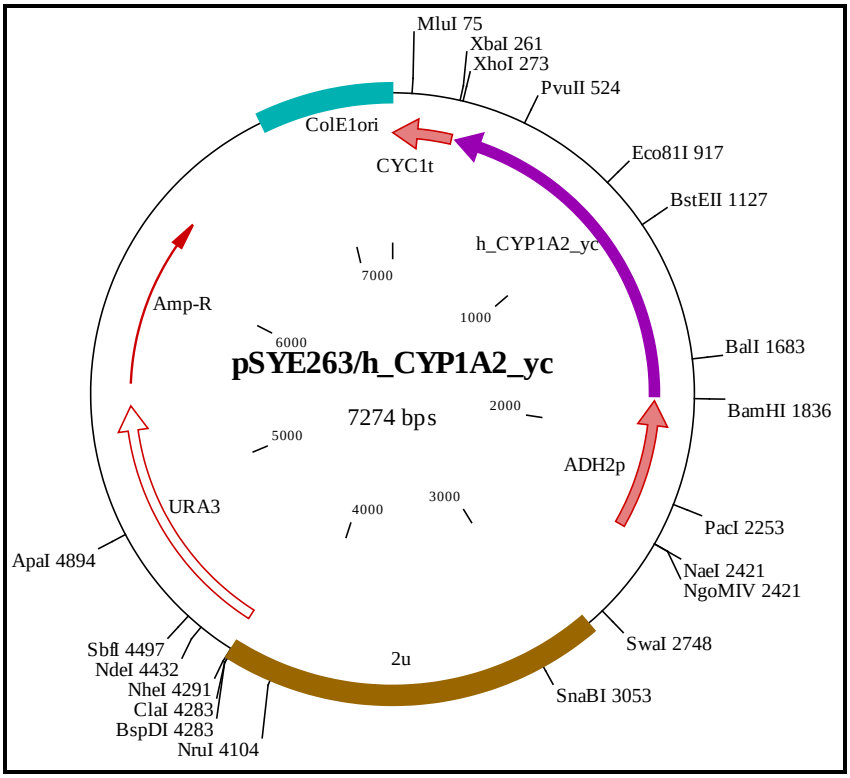


Figure 5.2. Episomal plasmid that contains a human CYP1A2 gene expression cassette. It was used to create the yeast strain YAB79::1A2(2µ). The restriction sites shown occur in the plasmid only once.

The *CYP1A2* gene, in the plasmid pSYE263/h_CYP1A2_yc, was synthesized using yeast biased codons. It is driven by the *ADH2* promoter which is upstream (5'-end) of the gene. The transcription terminator is from the yeast *CYC1* gene. The *CYP1A2* gene expression cassette consists of the *CYP1A2* gene sandwiched between the *ADH2* promoter and the *CYC1* terminator. The *URA3* gene is the dominant gene in yeast's biosynthetic pathway that leads to the synthesis of the nucleoside, uracil. It is the selection marker on the episomal plasmid. This allows selection (maintenance) of the plasmid when transformed into the cells of a yeast strain which lacks a functional *URA3* gene (represented by the term '*ura3*', in lowering casing). This implies that the episomal plasmid can continue to be maintained during growth when cells, containing the plasmid, are grown in minimal

SD medium that lacks uracil. The episomal plasmid is comprised of an essential part (1472 bp; '2u' in Figure 5.2) of the endogenous 2-micron (2u) sequence (6318 bp) of yeast that allows autonomous replication of the plasmid when yeast cells divide. The 1472 bp '2u' sequence in the plasmid, shown in Figure 5.2, includes the sequences that code for the Rep 1 and Rep 2 proteins that are involved in plasmid partitioning and stability during yeast cell division (Velmurugan et., 2000; Rizvi et al., 2017). It should be emphasised that there is no selection pressure in full YPD medium which contains all essential nutrients, including uracil. Cells from the strain YY7 when transformed with episomal plasmid were named YAB79::1A2(2µ).

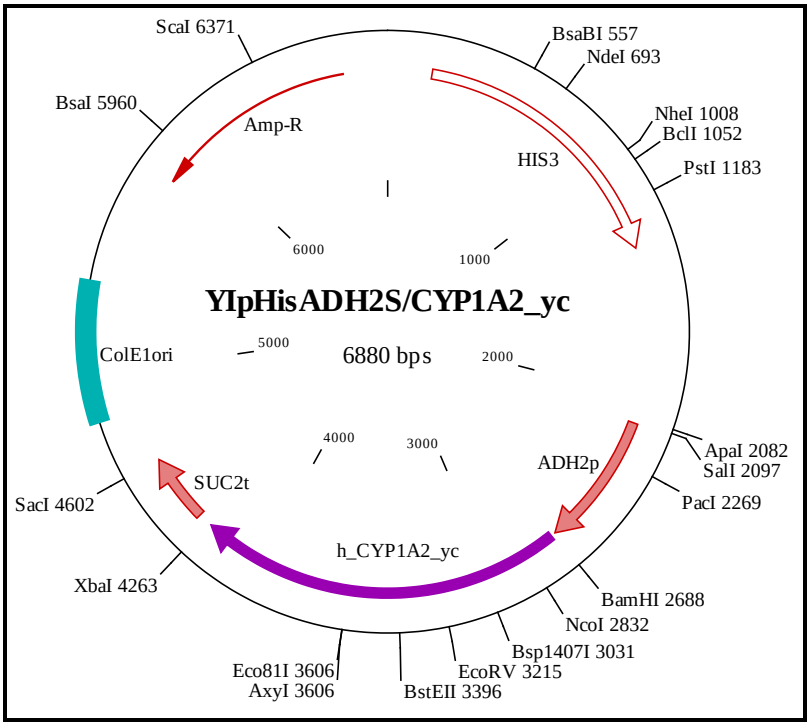


Figure 5.3. The plasmid that has been used for integration at the HIS3 locus of the yeast strain YAB79 that lacks a functional HIS3 gene ('his3'). The plasmid contains a functional copy of the HIS3 gene and was used to create the strain YAB79::1A2(HIS3). The restriction sites shown on the map occur in the plasmid only once.

For integration, the plasmid, in Figure 5.3, must be linearized at a restriction site within the *HIS3* gene that is encoded by the plasmid. The *HIS3* gene is a dominant gene in the yeast cells' pathway to making the amino acid histidine biosynthetically. The restriction site for linearization is chosen such that it occurs only once in the plasmid. This is to enable efficient homologous recombination of a linearized DNA fragment to occur on the chromosomal locus of yeast cells where the non-functional *his3* gene resides. Plasmid integration into the chosen *his3* chromosomal locus of the strain is confirmed by plating cells, after yeast transformation, in SD minimal medium that lacks histidine. Cells that grow in this medium must once again contain a functional *HIS3* gene. After thus confirming integration, the cells containing an integrated copy of the *CYP1A2* gene expression cassette can be propagated infinitely, without any selection, in full YPD medium.

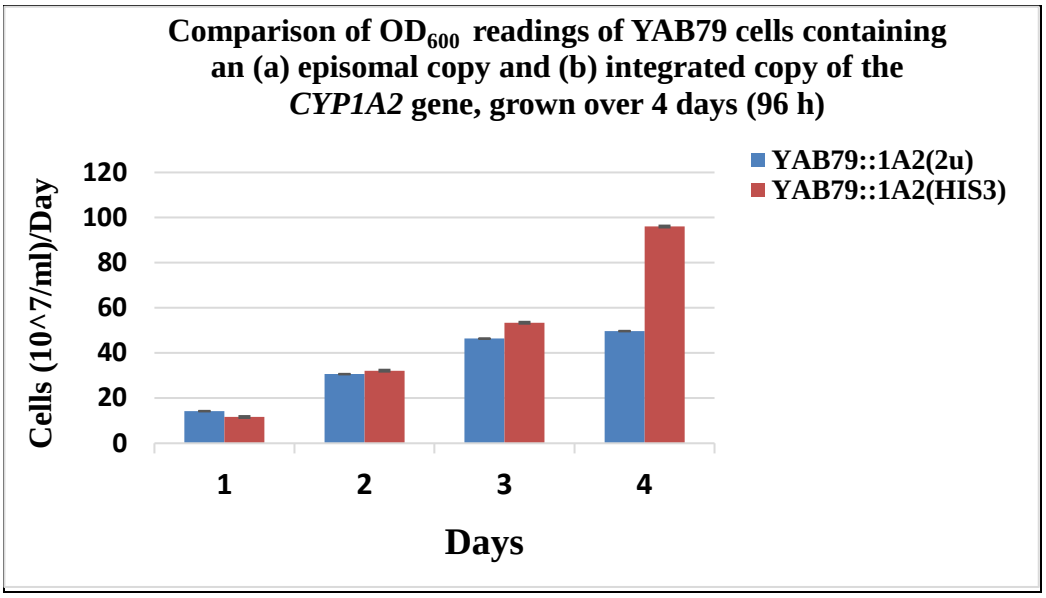


Figure 5.4. Comparison of the growth of YAB79 derivatives, harbouring (a) a CYP1A2 gene expression cassette-bearing episomal 2µ-plasmid [YAB79::1A2 (2µ)] and (b) and an identical expression cassette that had been integrated into the HIS3 locus of the yeast chromosome [YAB79::1A2 (HIS3)]. The optical densities were measured at 600 nm (cell count) and 1X 10^7 cells were taken for measurement in each case.The data represent mean ± S.D. of three independent experiments.

The bar plots in Figure 4 show the optical density, measured at 600 nanometres (i.e. OD_{600}), of cells from strains that express (a) *CYP1A2* gene from an episomal plasmid [strain YAB79::1A2(2µ)] and (b) an integrated copy [strain YAB79::1A2(HIS3)]. After Day 1, YAB79::1A2(2µ) cells (blue coloured bar in the Figure) compared well with YAB79::1A2(HIS3) cells. From Day 2, however, YAB79::1A2(HIS3) cells grew far quicker than YAB79::1A2(2µ), the cells that contain the episomal plasmid. At completion of Day 3, cells of both strains doubled their cell numbers, the amount of nutrients in the cell culture medium playing an important role. At completion of Day 4, YAB79::1A2(2µ) cells did not show any further increase in cell number while the integrated strain YAB79::1A2(HIS3) almost doubled the number of cells that were obtained after Day 3.

We then asked the question why the density of cells of the strain YAB79::1A2(2µ), bearing an episomal plasmid, lags behind the cells of strain YAB79::1A2(HIS3) that contains an integrated copy of *CYP1A2* gene, after 4 days of growth. It could be assumed that the majority of YAB79::1A2(2µ) cells would have lost the plasmid after 4 days of growth because they were gown in the non-selective YPD medium. It would seem that cells were stressed after loss of plasmid and, therefore, lagged in growth. This could perhaps be explained by the fact that the YAB79 strain was originally derived from a basic strain that was circle-zero, that is, it lacked the endogenous 2µ plasmid which all wild yeast cells contain. The endogenous 2µ plasmid sustains cell growth. Circle-zero cells grow much slower than circle-plus cells (Chen et al., 2005). The episomal plasmid, which has been used for these studies, contains part of the endogenous 2µ plasmid and, hence, episomal plasmids are often referred to as 2µ plasmids. In the context of growth, an episomal plasmid is likely to compensate for the absence of the endogenous 2µ

plasmid. Therefore, it would be expected that, after loss of the *CYP1A2* gene-bearing episomal plasmid, during growth in non-selective YPD medium, the yeast cells would grow slower.

The observation that cells, containing an integrated copy of a human gene, grew so well over 4 days (i.e. up to an OD_{600} of around 100) has relevance to industrial scale production of recombinant CYP proteins in yeast. It would seem that another advantage of the YAB79 cells used is their robustness because of their ability to withstand high ethanol concentrations. Usually, most laboratory yeast strains die or grow very sluggishly in cell culture medium that contains ethanol. It should be highlighted that in each new consecutive cycle of growth in YPD medium, the cells were exposed to increasing concentrations of ethanol. Hence, cells containing integrated copies of genes would be extremely important as part of a generic manufacturing process whereby proteins such as CYP enzymes and other recombinant proteins are produced. Having cells growing to such high density, without any loss of genetic information from within the cells, would definitely allow consistent and reproducible production of high levels of CYP proteins.

Having obtained these results, it was thought that it would be interesting to find out if the integrated strain YAB79::1A2 (HIS3) produces more or less CYP enzyme activity than the episomal strain YAB79::1A2(2µ), per unit number of cells.

Figures 5.5 and 5.6 show that the yeast strain YAB79::1A2(2µ), containing the *CYP1A2* gene-bearing episomal plasmid, had a dramatic decrease in CYP1A2 enzyme activity, per 1X 10^7 cells, compared to cells that contained an integrated copy of the *CYP1A2* gene, that is, YAB79::1A2(HIS3). Since the cells were not subjected to any selection pressure in YPD medium, as explained above, YAB79::1A2 (2µ) cells had lost the ability to

sustain the *CYP1A2* gene bearing plasmid. As a result of the instability of the episomal plasmid in YPD, CYP1A2 enzyme activity decreased drastically compared to [YAB79::1A2 (HIS3)] cells that contained an integrated copy of the *CYP1A2* gene.

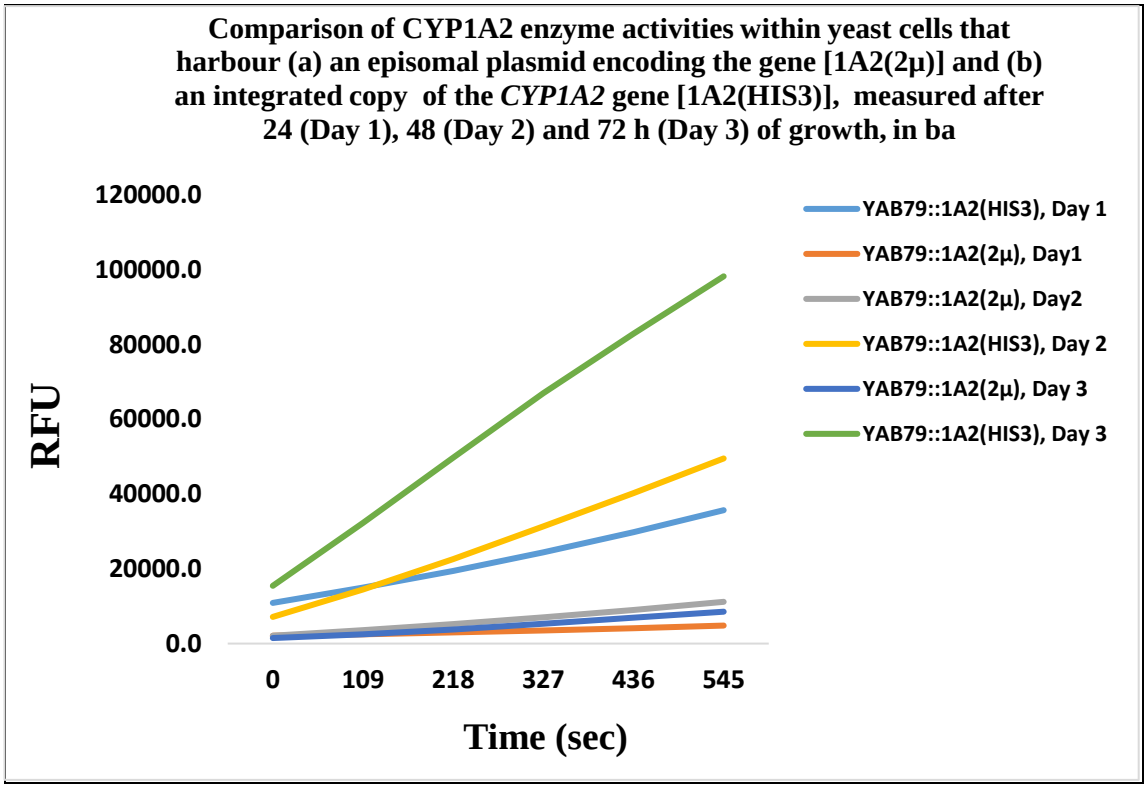


Figure 5.5. Comparison of the kinetics of CYP1A2 enzyme activities obtained from YAB79::1A2(2µ) and YAB79::1A2(HIS3) cells. Activities were monitored in 1X 10^7 cells that were grown for 1 to 3 days, using CEC as a substrate (Table 1). The depicted results were an average of three independent experiments.

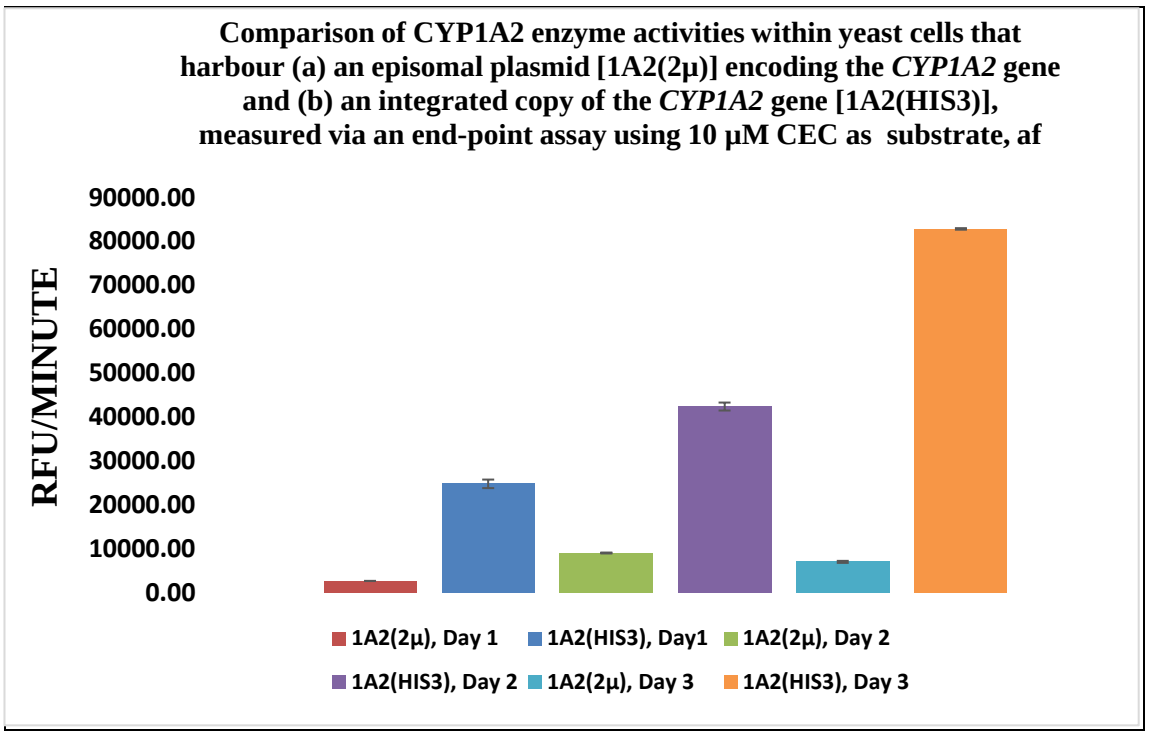


Figure 5.6. Comparison of CYP1A2 enzyme activities obtained from YAB79::1A2(2µ) and YAB79::1A2(HIS3) cells. Activities were determined at 9 min, using 1X 10^7 cells, in an end-point enzymatic assay. CEC was used as the fluorogenic substrate (Table 1). The Figure mirrors the results shown in Figure 5.5.

The results in Figures 5.5 and 5.6 depict the strength of CYP1A2 activities displayed by strains that express an integrated copy of the gene. The episomal plasmid strain YAB79::1A2 (2µ) has the highest activity on Day 2 while there was a sharp decrease in the activity of episomal on Day 3. The strain YAB79::1A2 (HIS3), containing the integrated copy of *CYP1A2* gene, show gradual increase in activity from Days 1 to 3. Activity increased only ~30% from Days 1 to 2 but there was ~98 % increase from Days 2 to 3. However, it was most surprising that the strain expressing *CYP1A2* from an episomal would produce much less CYP enzyme than the strain that contains a single copy of the *CYP1A2* gene even on Day 1. Episomal plasmids, containing the 2-micron (2µ) sequence should, in theory, have produced multiple copies (i.e. 40-60 copies) of the

gene per cell (Broach et al., 1991; Futcher and Cox, 1993; Falcon et al., 2005). However, during the expression of toxic human proteins in yeast, the copy number of a 2µ-plasmid could be less than one (Kingsman et al., 1985). CYP-CPR complexes constitute redox enzymes. Overexpression of such complexes could potentially be very harmful for living cells. In human cells, CYP expression is highly regulated. When expressed, their levels are very low and that is one of the reasons that the availability of recombinant CYP enzymes is so crucial for drug discovery. Moreover, overexpression of CPR in humans is known to cause neurodegenerative disease (Yao *et al.,* 2013). Hence, overexpression of CPR in human cells is toxic.

It was, therefore, very exciting to observe that the yeast strain YAB79::1A2(HIS3), containing 'one' integrated copy of the *CYP1A2* gene, not only grows to a much higher optical density (Figure 5.4) but also that, per defined number of cells (e.g. 1X 10^7), the strain expresses a lot more enzyme than the strain YAB79::1A2(2µ) which contains a *CYP1A2* gene bearing episomal, 2µ-plasmid which ought to produce multiple copies of *CYP1A2* gene. This would indicate that integration of *CYP* gene expression cassettes into yeast's chromosomes may provide an excellent method for stable production of human CYP enzymes at high levels.

5.3.3 Comparison of CYP1A2 enzyme activity produced from a yeast strain that expresses 2 copies of the CYP1A2 gene and strains that express 1 copy of the gene

A second copy of the *CYP1A2* gene expression cassette was introduced into the *URA3* locus of the strain YAB79::1A2(HIS3) to obtain the new strain YAB79::1A2(HIS3,URA3) [1A2(HIS3,URA3) in Figure 5.7] which contains 2 copies of

the *CYP1A2* gene. Activity produced in this 2-copy *CYP1A2* strain was then compared with the ones produced by the strains that express only one copy of *CYP1A2* gene from two different chromosomal loci, *HIS3* and *URA3* [strains 1A2(HIS3) and 1A2URA3) in Figure 5.7]. The results are depicted Figures 5.7 and 5.8. Equal numbers of cells (1X 10^7) were used for determination of CYP1A2 enzyme activity expressed by each strain.

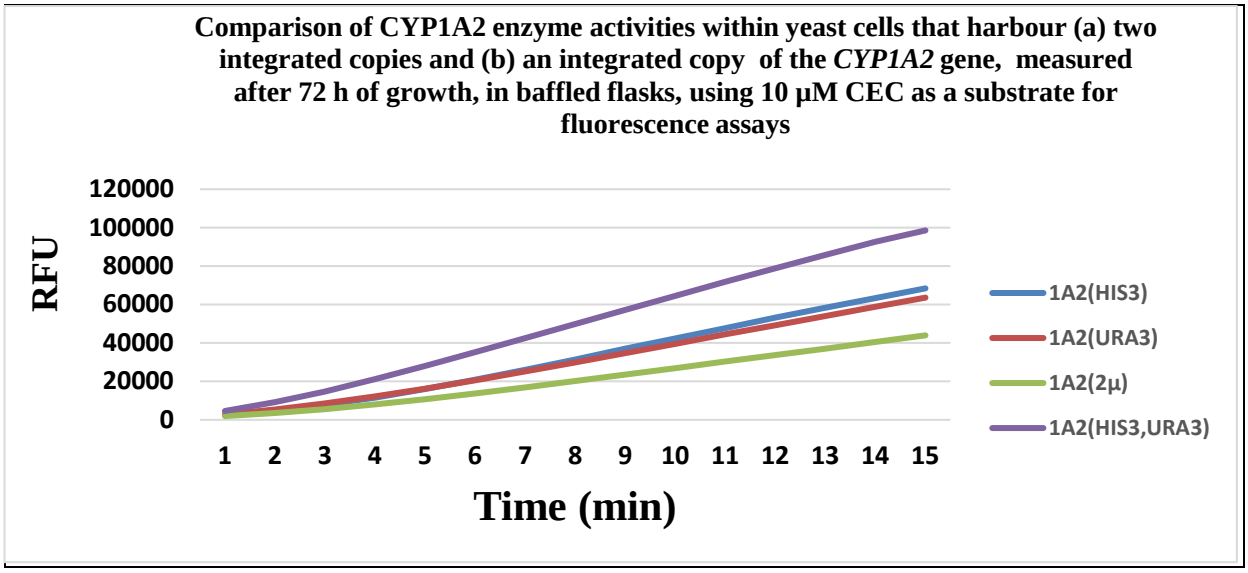


Figure 5.7. Comparison of the kinetics of CYP1A2 enzyme activities obtained from the yeast strain YAB79::1A2(HIS3,URA3), that contains 2 copies of CYP1A2 gene, and strains [YAB79::1A2(HIS3) & YAB79::1A2(URA3)] that contain one copy of the gene at the HIS3 and URA3 loci. The strain YAB79::1A2(2µ), that contained the episomal plasmid, was used as a control. Activities were determined using 1X 10^7 cells from cultures that were grown for 3 days. CEC was used as a substrate. The depicted results were an average of three independent experiments.

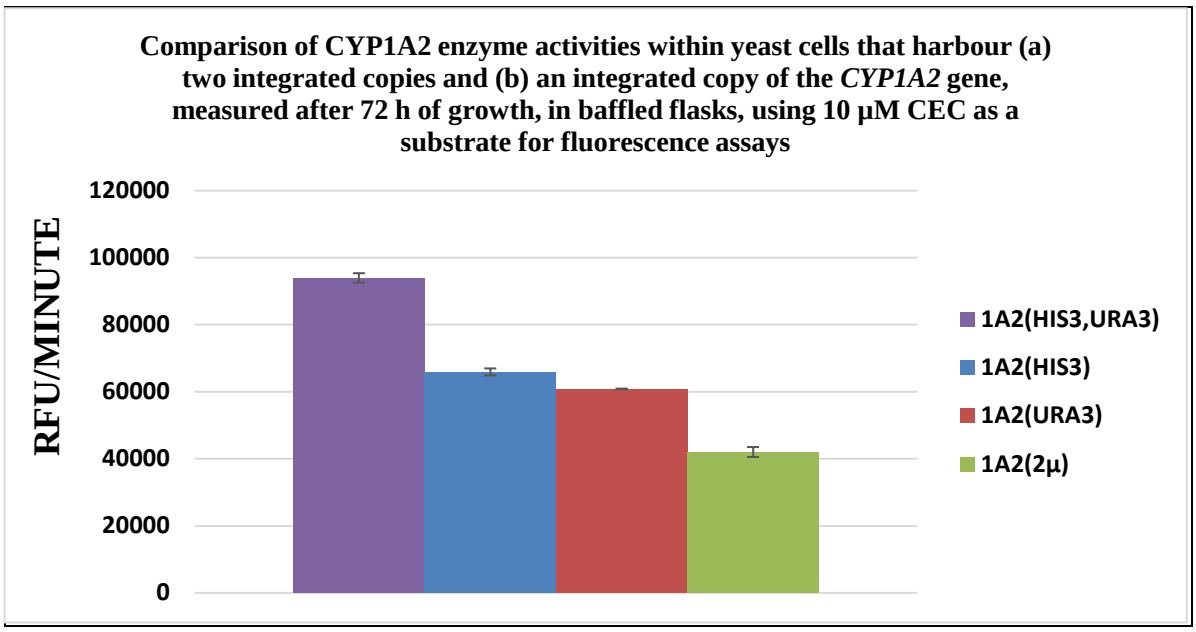


Figure 5.8. Comparison of CYP1A2 enzyme activities obtained from the 2-copy [YAB79::1A2(HIS3,URA3)] yeast strain and 1-copy CYP1A2 expressing strains [YAB79::1A2(HIS3) and YAB79::1A2(URA3)]. YAB79::1A2(2µ) cells were used as control. Activities were determined at 15 min, using 1X 10^7 cells, in an end-point enzymatic assay, using CEC as a substrate.

The graph in Figure 5.7 and the bar plot in Figure 5.8 depict the expression of CYP1A2 in the strain YAB79 which contains the modified human P450 reductase ΔhRDM at the *LEU2* locus and cytochrome *b5* at the *TRP1* locus. It is observed that the strain expressing 2 copies of the *CYP1A2* gene, simultaneously from the *HIS3* and *URA3* loci, is by far the best in producing the CYP1A2 enzyme, compared to the strains that express one copy of the *CYP1A2* gene. Both the *HIS3* and *URA3* loci were chosen for integration since it had been found, from the experiments described in Chapter 4, that these loci produced the highest amount of CYP enzymes. As seen before, expression of the enzyme from an episomal plasmid once again produced the least amount of CYP enzyme.

5.3.4 Comparison of amounts of CYP1A2 microsomal enzyme isolated from yeast strains containing CYP1A2 gene which is (a) chromosomally integrated at two different loci and (b) encoded by an episomal plasmid

Until now, the amounts produced within whole cells were inferred from the CYP enzyme activities that had been measured within whole cells. Although indicative, this was not a precise way of quantifying the amounts produced. Hence, microsomal enzymes were isolated from the 2-copy strain YAB79::1A2(HIS3,URA3) [referred to as 1A2(HIS3,URA3) in Figure 5.9] and the episomal strain, YAB79::1A2(2μ) [1A2(2μ), in Figure 5.9] and the amounts produced from 400 ml of cell culture were compared.

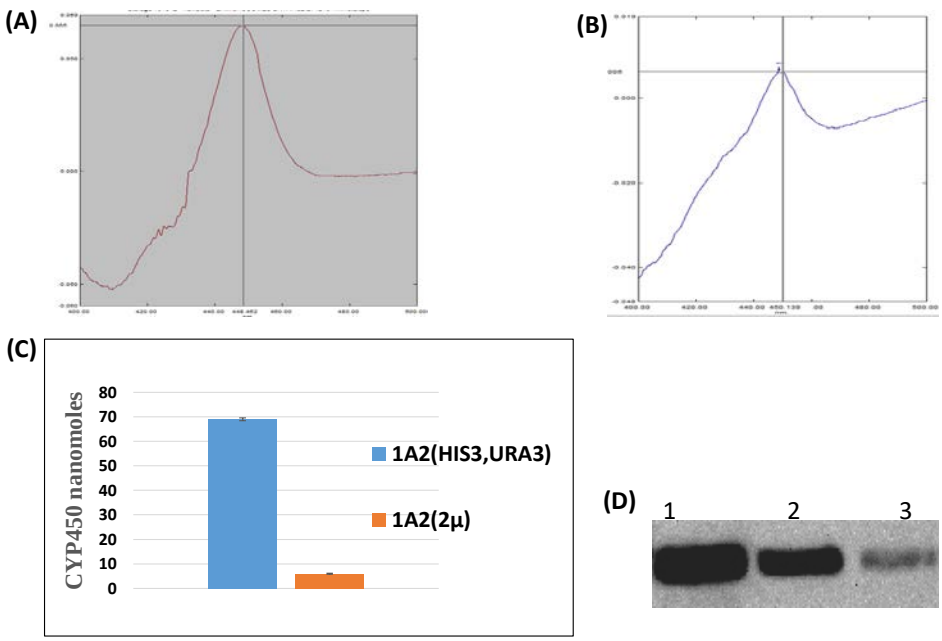


Figure 5.9. (A), (B): CYP450 contents, in the strains YAB79::1A2(HIS3,URA3) (A) and YAB79::1A2(2μ) (B) were determined by Fe^{2+} versus Fe^{3+} CO difference spectrophotometry using 1.0 μg of microsomal protein. (C) The amount of CYP450 (in nanomoles), obtained from the 2 strains (400 ml of YPD culture grown in baffled flasks), and represented as bar plots. The data represent mean ± S.D. of three independent experiments. (D) Western blots used 10 μg of total protein from the strains YAB79::1A2 (HIS3, URA3) (lane 1), YAB79::1A2 (HIS3) (lane 2) and YAB79::1A2 (2μ) (lane 3) to probe with a CYP1B1 specific antibody (Santa Cruz Biotechnology, sc-30085).

The microsomal CYP1A2 enzymes isolated from the two strains were measured via carbon monoxide (CO) difference spectroscopy (see Chapter 2, Section 2.8.3.1) which involves binding of CO to CYP450 enzymes [Figure 5.9 (A) and (B)]. Figure 5.9 (C) shows that 400 ml of cells cultured from the integrated strain YAB79::1A2(HIS3,URA3) produced 69 nM (i.e. 69 nanomoles) of microsomal CYP1A2 enzyme compared to only 6 nM of enzyme from 400 ml of cells from the strain YAB79::1A2(2μ). The Western blot in Figure 5.9 (D) shows a clear difference in the amounts of CYP1A2 produced within the cells from the strains

(a) YAB79::1A2(HIS3,URA3), harbouring 2 copies of *CYP1A2* gene,

(b) YAB79::1A2(HIS3), harbouring 1 copy of *CYP1A2* gene, and

(c) YAB79::1A2(2μ), harbouring an episomal plasmid that encodes the *CYP1A2* gene.

All Western blot protein bands, in this Chapter, were visualized using the software Quantity One (Chem Doc; Bio-Rad). The strain YAB79::1A2(HIS3,URA3) expressing 2 copies of the *CYP1A2* gene produced the highest amount of CYP1A2 protein.

5.3.5 Comparison of human CYP3A4 enzyme activities, expressed within cells, from yeast strains that contain 1-3 integrated copies of CYP3A4 gene

With the aim of finding a yeast strain that would produce high amounts of human CYP3A4 enzyme, attempts were made to express the enzyme from different yeast strains that contain different copy numbers of a human *CYP3A4* gene expression cassette. The

human *CYP3A4* gene was chemically synthesised using yeast biased codons. Different yeast strains were created for comparative analysis of human CYP3A4 enzyme expression.

YAB79 cells (containing *ΔhRDM* and cytochrome *b5* genes) were transformed with an

(1) Episomal (pSYE263/h_CYP3A4_yc; Figure 3.6) to create a control strain,

(2) Integrating plasmid (YIpAdeADH2S/CYP1A2_yc; Figure 4.18, Chapter 4),

(3) Integrating plasmid (YIpHisADH2S/CYP1A2_yc; Figure 4.24, Chapter 4),

(4) Integrating plasmid (YIpUraADH2S/CYP1A2_yc; Figure 4.21, Chapter 4),

The resultant strains obtained after transformation were named

(i) YAB79::3A4(2µ), a control strain, where the plasmid replicates extra-chromosomally during cell division occurring during yeast cell growth [referred to as 3A4(2µ) in Figure 5.10],

(ii) YAB79::3A4(ADE2) [referred to as 3A4(ADE2) in Figure 5.10],

(iii) YAB79::3A4(HIS3) [referred to as 3A4(HIS3) in Figure 5.10],

(iv) YAB79::3A4(URA3) [referred to as 3A4(URA3) in Figure 5.10].

The strains YAB79::3A4(ADE2) and YAB79::3A4(HIS3) were used to introduce a second copy of the human *CYP3A4* gene. The resultant strains obtained after transformation were named

(v) YAB79::3A4(ADE2,HIS3) [referred to as 3A4(ADE2,HIS3) in Figure 5.10],

(vi) YAB79::3A4(ADE2,URA3) [referred to as 3A4(ADE2,URA3) in Figure 5.10],

(vii) YAB79::3A4(HIS3,URA3) [referred to as 3A4(HIS3,URA3) in Figure 5.10],

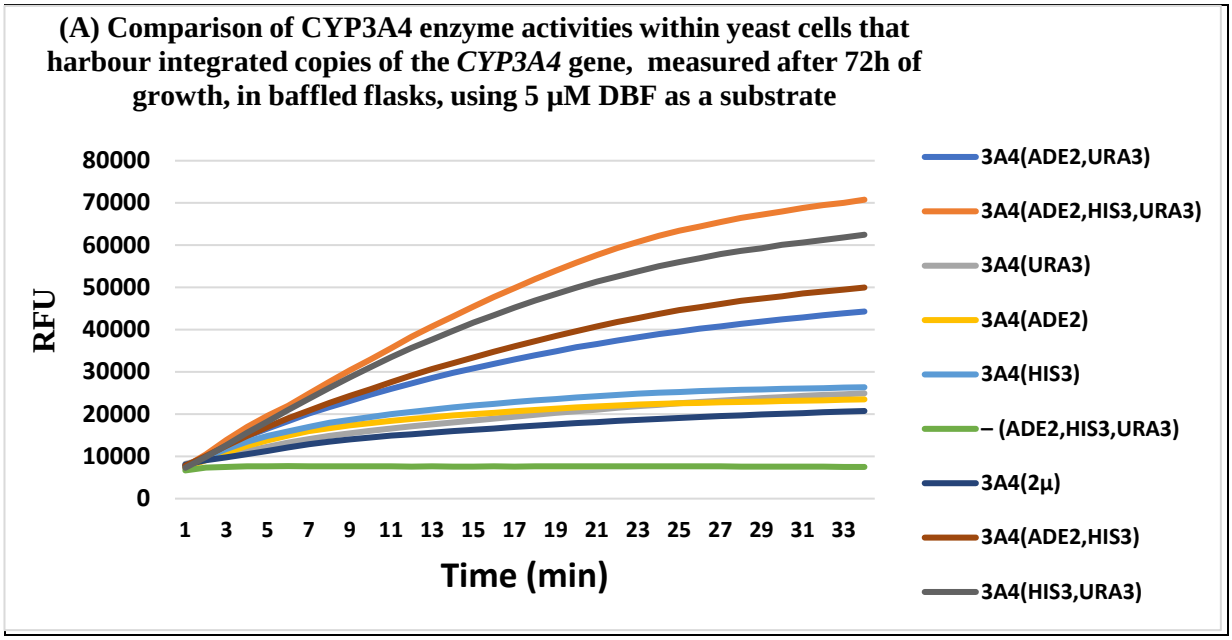
To the yeast strain YAB79::3A4(HIS3,URA3) was introduced a third copy of the *CYP3A4* gene to create the strain

(viii) YAB79::3A4(ADE2,HIS3,URA3) [referred to as 3A4(ADE2,HIS3,URA3) in Figure 5.10].

The strain YAB79:: – (ADE2,HIS3,URA3) was generated to act as a negative control. It was created by stepwise integration of an empty plasmid that does not contain any *CYP3A4* gene. In Figure 5.10, it is referred to simply as "– (ADE2,HIS3,URA3)".

All integrating plasmids, listed above, allow integration of a plasmid into yeast's chromosomes.

The yeast strains were grown in YPD medium for 72 h, replenished with new medium every 24 h. The CYP3A4 activities, within cells, were monitored in ($1X\ 10^7$) cells harvested after 72 h. Each culture was started with an inoculum of equal number of cells. All cell cultures were grown exactly in the same way, with fresh YPD medium being provided at identical time points of growth. The results in Figure 5.10 show comparative CYP3A4 enzyme activities obtained from different strains.



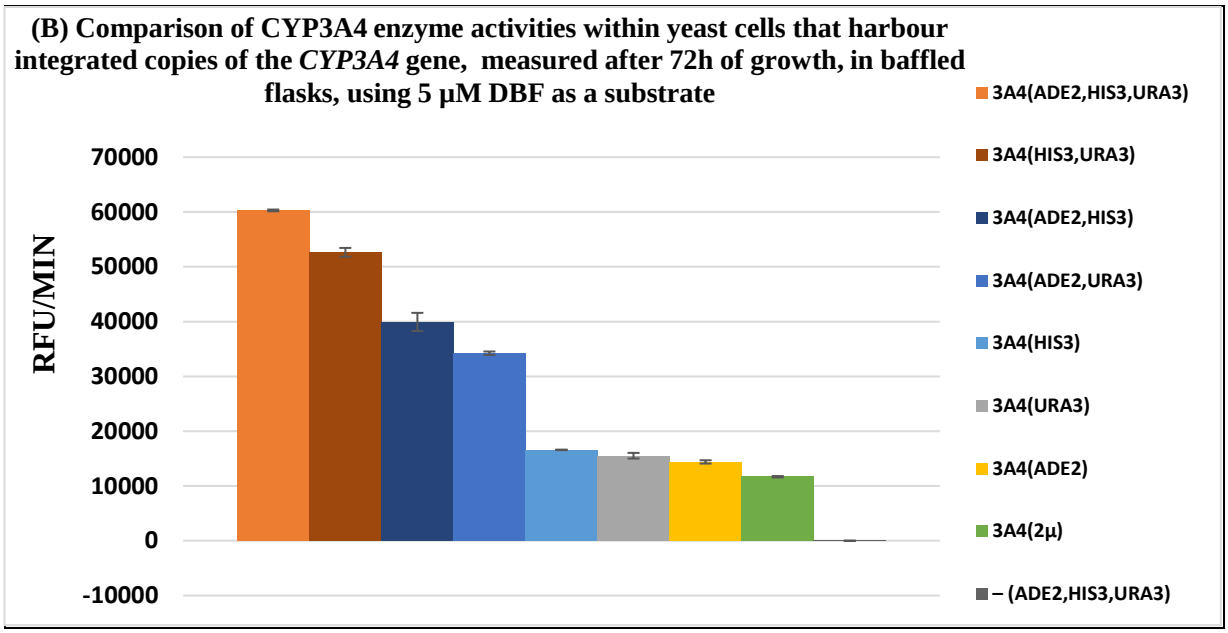


Figure 5.10. The graph (A) shows the comparison of CYP3A4 enzyme activities within cells of different yeast strains, derived from YAB79. They contain the CYP3A4 gene in 1 copy, 2 & 3 integrated copies, and CYP3A4 gene encoded by an episomal plasmid. A strain which contained 3 empty plasmids on the ADE2, HIS3 and URA3 loci acted as a negative control. The graphs represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

The results in Figure 5.10 clearly show that the yeast cells from the strain YAB79::3A4(ADE2,HIS3,URA3) [referred to as 3A4(ADE2,HIS3,URA3), containing 3 copies of the human *CYP3A4* gene, produce the highest activity of CYP3A4 enzyme implying. Hence, it could be deduced that the 3-copy strain, per 1X 10^7 cells, produces the highest amount of the CYP3A4 enzyme. However, the 2-copy strain, expressing CYP3A4 simultaneously from the *HIS3* and *URA3* chromosomal loci, was a close second. Since the 3-copy strain did not grow as well as the 2-copy strain, probably because of latent toxicity due to overexpression, the latter [i.e. the strain YAB79::3A4(HIS3,URA3), referred to as 3A4(HIS3,URA3)], was used further for isolation of microsomal CYP3A4 enzyme.

5.3.6 Comparison of amounts of CYP3A4 microsomal enzyme isolated from yeast strains containing CYP3A4 gene which is (a) chromosomally integrated at two different loci and (b) encoded by an episomal plasmid

In order to evaluate the true expression levels of CYP3A4 enzyme in the strain YAB79::3A4(HIS3,URA3), referred to as 3A4(HIS3,URA3) in Figure 5.11, microsomal CYP3A4 enzyme was isolated. The correctly folded CYP protein, which provides the activity, was determined at 450 nm via CO difference spectroscopy (see Chapter 2, Section 2.8.3.1). Results shown in panels (A) and (B) of Figure 5.11 clearly show that the amount of enzyme produced by this integrated strain is much more than the enzyme obtained from the episomal plasmid containing strain YAB79::3A4(2µ), referred to as

3A4(2µ) in Figure 5.11. Both strains were grown in 400 ml of YPD cell culture, under exactly the same conditions.

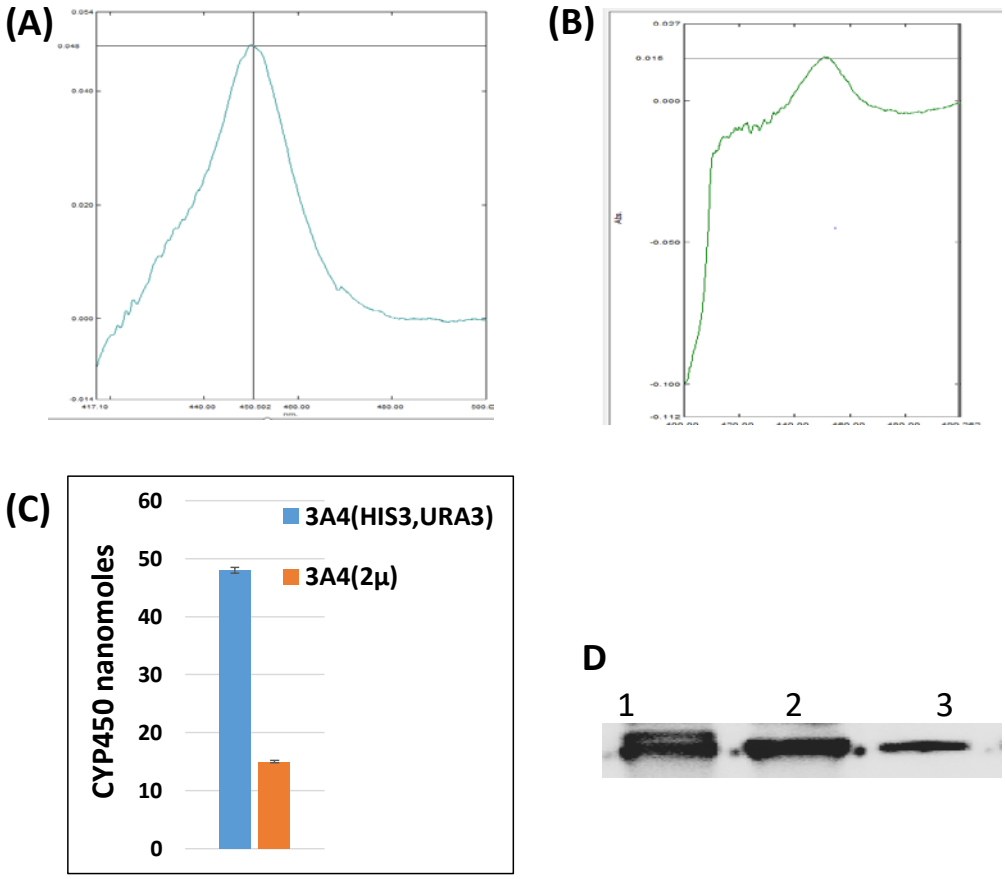


Figure 5.11. (A), (B): P450 contents, in the strains YAB79::3A4(HIS3,URA3) [3A4(HIS3,URA3); (A)] and YAB79:: 3A4(2µ) [3A4(2µ); (B)] were determined by CO difference spectroscopy using 1.0 µg of microsomal protein. (C) The amount of CYP450 (in nanomoles), obtained from the 2 strains (400 ml of YPD culture grown in baffled flasks), and represented as bar plots. The data represent mean ± S.D. of three independent experiments. (D) Western blots where 10 µg of total protein from the strains YAB79::3A4(HIS3,URA3) (lane 1), YAB79::3A4(HIS3) (lane 2) and YAB79::3A4(2µ) (lane 3) were probed with a CYP3A4 specific antibody (Santa Cruz Biotechnology, Cat no:sc-27639).

Panel C (Figure 5.11) shows that the strain containing 2 integrated copies of the CYP3A4 gene produced 48 nanomoles of CYP3A4 enzyme while the episomal produced only 15 nanomoles from 400 ml of cell culture. Panel D depicts a Western blot of CYP3A4 proteins obtained from different strains. It visually confirms that the strains that express

1-2 integrated copies of *CYP3A4* gene produce more protein than the strain that expresses *CYP3A4* from an episomal plasmid.

5.3.7 Comparison of human CYP3A5 enzyme activities, expressed within yeast cells, from strains that contain 1 copy and 2 integrated copies of CYP3A5 gene

With the aim of identifying a yeast strain that would produce high amounts of human CYP3A5 enzyme, four new strains were created from the strain YAB79 that contains *ΔhRDM* and cytochrome *b5* genes at the *LEU2* and *TRP1* loci, respectively.

YAB79 cells were transformed with an

(1) Episomal (pSYE263/h_CYP3A5_yc; Figure 3.92, Chapter 3) to create a control strain,

(2) Integrating plasmid (YIpHisADH2S/CYP3A5_yc; Figure 4.77, Chapter 4),

(3) Integrating plasmid (YIpUraADH2S/CYP3A5_yc; Figure 4.79, Chapter 4).

The resultant strains obtained after transformation were named

(i) YAB79::3A5(2µ), the control strain, [referred to as 3A5(2µ) in Figure 5.12],

(ii) YAB79::3A5(HIS3) [referred to as 3A5(HIS3) in Figure 5.12], and

(iii) YAB79::3A5(URA3) [referred to as 3A5(URA3) in Figure 5.12].

The strain YAB79::3A5(HIS3) was used to introduce a second copy of the human *CYP3A5* gene at the *URA3* locus. The resultant strain obtained after transformation was named YAB79::3A5(HIS3,URA3), referred to as 3A5(HIS3,URA3) in Figure 5.12.

The yeast strains were grown in YPD medium for 72 h, replenished with new medium every 24 h. The CYP3A5 activities, within cells, were monitored in (1X 10^7) cells harvested after 72 h of growth. Each culture was started with an inoculum of equal number of cells. All cell cultures were grown exactly in the same way, with fresh YPD medium being provided at identical time points of growth. The results in Figure 5.12 show comparative CYP3A5 enzyme activities obtained from four different strains.

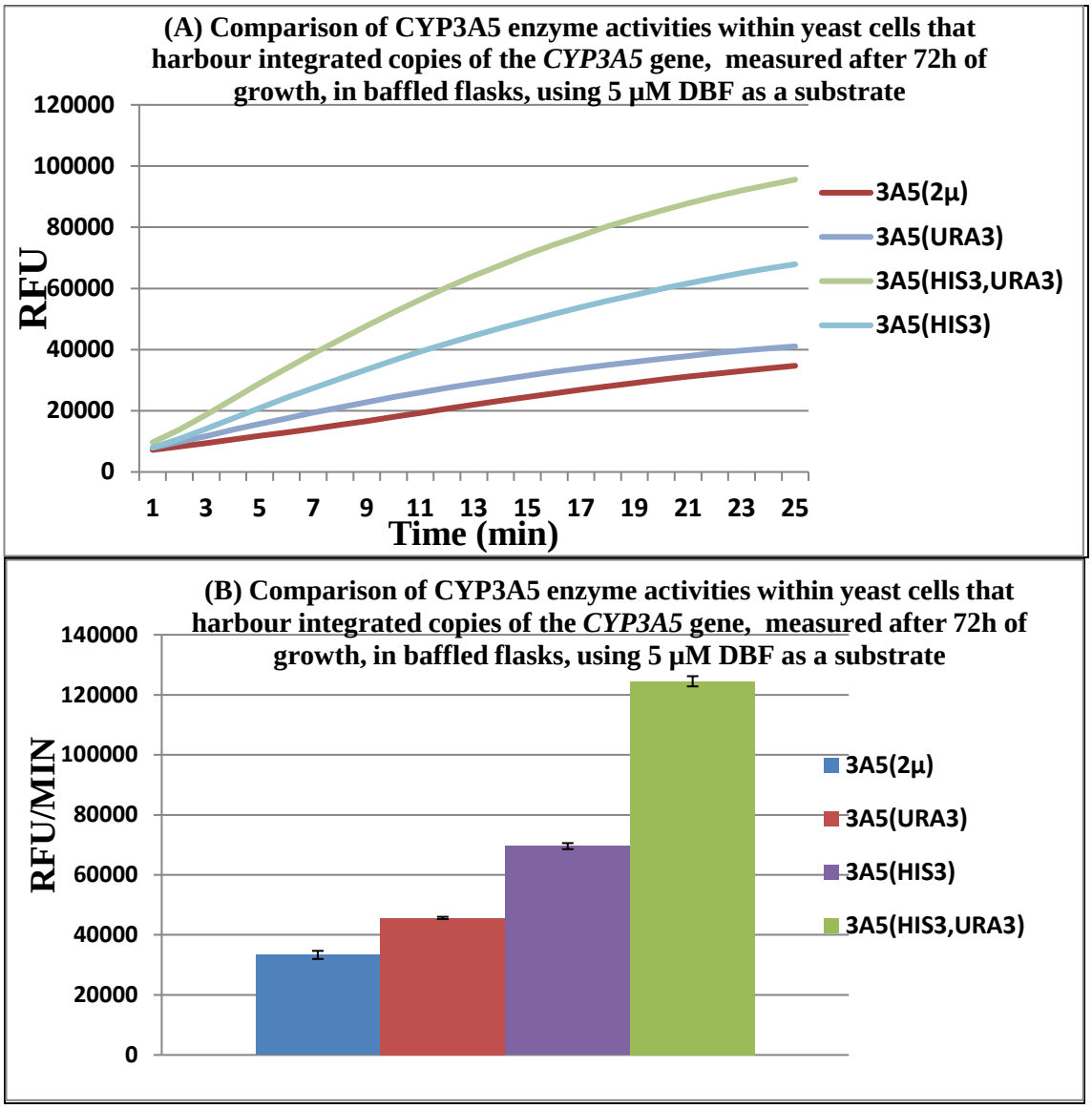


Figure 5.12. The graph (A) shows the comparison of CYP3A5 enzyme activities within cells of different yeast strains, derived from YAB79. They contain the CYP3A5 gene in 1 integrated copy, 2 integrated copies, and the CYP3A5 gene encoded by an episomal plasmid. The graphs (A) represent the average of

results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

Figure 5.12 depicts the expression of human CYP3A5 in baker's yeast. The results show that the 2-copy integrated strain YAB79::3A5(HIS3,URA3), referred to as 3A5(HIS3,URA3), has the highest activity. This strain was used to prepare CYP3A5 microsomal enzyme.

5.3.8 Comparison of amounts of CYP3A5 microsomal enzyme isolated from yeast strains containing CYP3A5 gene which is (a) chromosomally integrated at two different loci and (b) encoded by an episomal plasmid

In order to evaluate the true expression levels of CYP3A5 enzyme in the strain YAB79::3A5(HIS3,URA3), referred to as 3A5(HIS3,URA3) in Figure 5.13, microsomal CYP3A4 enzyme was isolated. Both strains were grown in 400 ml of YPD cell culture, under exactly the same conditions. The correctly folded CYP protein, which provides the activity, was determined at 450 nm via CO difference spectroscopy (see Chapter 2, Section 2.8.3.1). Results shown in panels (A) and (B) of Figure 5.13 clearly show that the amount of enzyme produced by this integrated strain is more than the enzyme obtained from the episomal plasmid containing strain YAB79::3A5(2µ), referred to as 3A5(2µ) in Figure 5.13. Comparison of the graphs in panels (A) and (B) reveals that the amounts of cytochrome b5 expressed is much less in YAB79::3A5(HIS3,URA3) cells than in the strain YAB79::3A5(2µ). This may imply that the overall CYP3A5 microsomal enzyme activity from YAB79::3A5(HIS3,URA3) may be lower than the enzyme obtained from

YAB79::3A5(2µ), if cytochrome b5 had a role in the overall activity of CYP3A5. However, it has been shown later in this Chapter, after isolation of the microsomal enzyme, cytochrome b5 levels may not be too relevant for CYP3A5 activity. This is in contrast to what has been published earlier (Yamazaki et al., 2002).

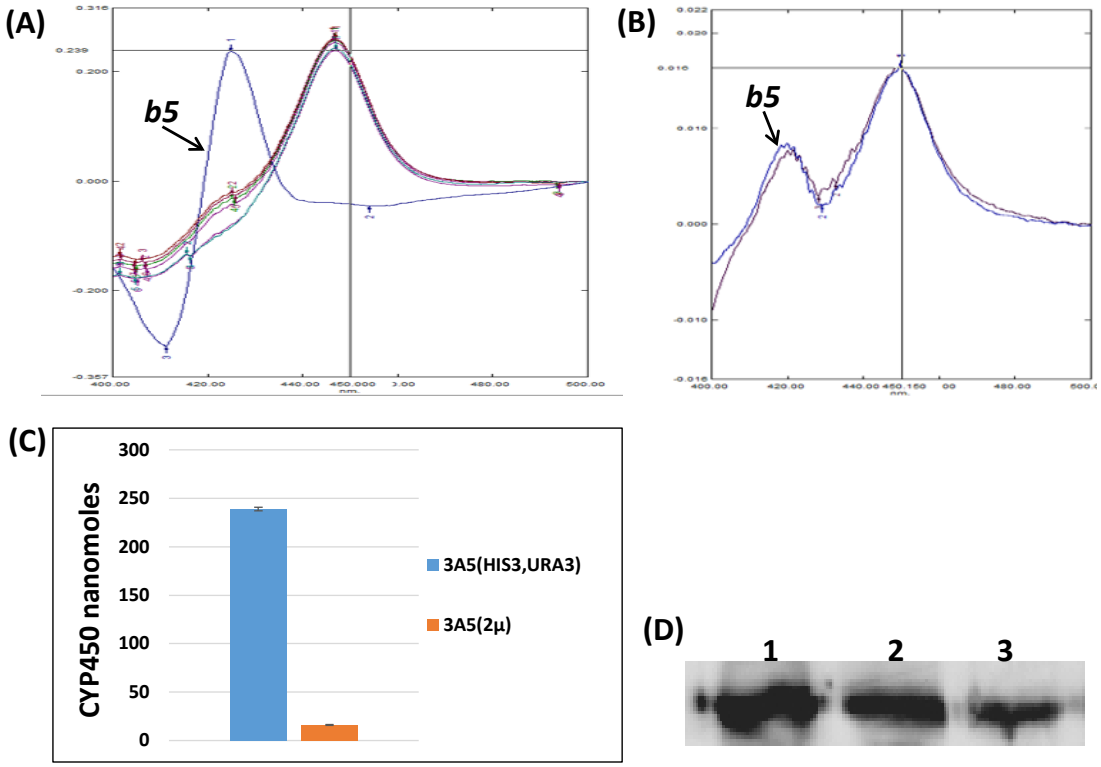


Figure 5.13. (A), (B): P450 contents, in the strains YAB79::3A5(HIS3,URA3) [3A5(HIS3,URA3); (A)] and YAB79::3A5(2µ) [3A5(2µ); (B)] were determined by CO difference spectroscopy using 1.0 µg of microsomal protein. (C) The amount of CYP450 (in nanomoles), obtained from the 2 strains (from 400 ml of YPD culture grown in baffled flasks), and represented as bar plots. The data represent mean ± S.D. of three independent experiments. (D) Western blots where 10 µg of total protein from the strains YAB79::3A5(HIS3,URA3) (lane 1), YAB79::3A5(HIS3) (lane 2) and YAB79::3A5(2µ) (lane 3) were probed with a CYP3A5 specific antibody (Santa Cruz Biotechnology, Cat no:sc-53616).

Panel C (Figure 5.13) shows that the strain containing 2 integrated copies of the CYP3A5 gene produced 245 nanomoles of *CYP3A5* enzyme while the episomal produced only 15 nanomoles from 400 ml of cell culture. Panel D depicts a Western blot of CYP3A5 proteins obtained from different strains. One can plainly see that the strains which express

1-2 integrated copies of human *CYP3A5* gene produce more protein than the strain that expresses *CYP3A5* from an episomal plasmid.

5.3.9 Comparison of human CYP2D6(1) (Val^{374}) enzyme activities, expressed within yeast cells, from strains that contain 1 copy and 2 integrated copies of CYP2D6 gene

To find a yeast strain that would produce high amounts of human CYP2D6(1) enzyme [CYP2D6(Val^{374}); see Chapter 4, Section 4.6.1], four new strains were created from the strain YY7 that contains only *ΔhRDM* at the *LEU2* locus.

YY7 cells were transformed with an

(1) Episomal (pSYE263/h_CYP2D6(1)_yc; Figure 3.38, Chapter 3) to create a control strain,

(2) Integrating plasmid (YIpHisADH2S/CYP2D6(1)_yc; Figure 4.43, Chapter4),

(3) Integrating plasmid (YIpUraADH2S/CYP2D6(1)_yc; Figure 4.41, Chapter 4).

The resultant strains obtained after transformation were named

(i) YY7::2D6-1(2μ), the control strain, [referred to as 2D6-1(2μ) in Figure 5.14],

(ii) YY7::2D6-1(HIS3) [referred to as 2D6-1(HIS3) in Figure 5.14], and

(iii) YY7::2D6-1(URA3) [referred to as 2D6-1(URA3) in Figure 5.14].

A second copy of the human *CYP2D6(1)* gene was introduced at the *URA3* locus, via transformation, into the strain YY7::2D6-1(HIS3). The resultant strain was named YY7::2D6-1(HIS3,URA3), referred to as 2D6-1(HIS3,URA3) in Figure 5.14.

The yeast strains were grown in YPD medium for 72 h, replenished every 24 h with new medium. The CYP2D6-1 [i.e. CYP2D6(Val^{374})] enzyme activities, within cells, were

monitored in (1X 10^7) cells harvested after 72 h of growth. Each culture was grown identically in YPD medium with an initial inoculum of equal number of cells. Fresh YPD medium was provided at each 24 h cycle of growth. The results in Figure 5.14 show the CYP2D6(1) (Val^{374}) enzyme activities obtained in four different strains.

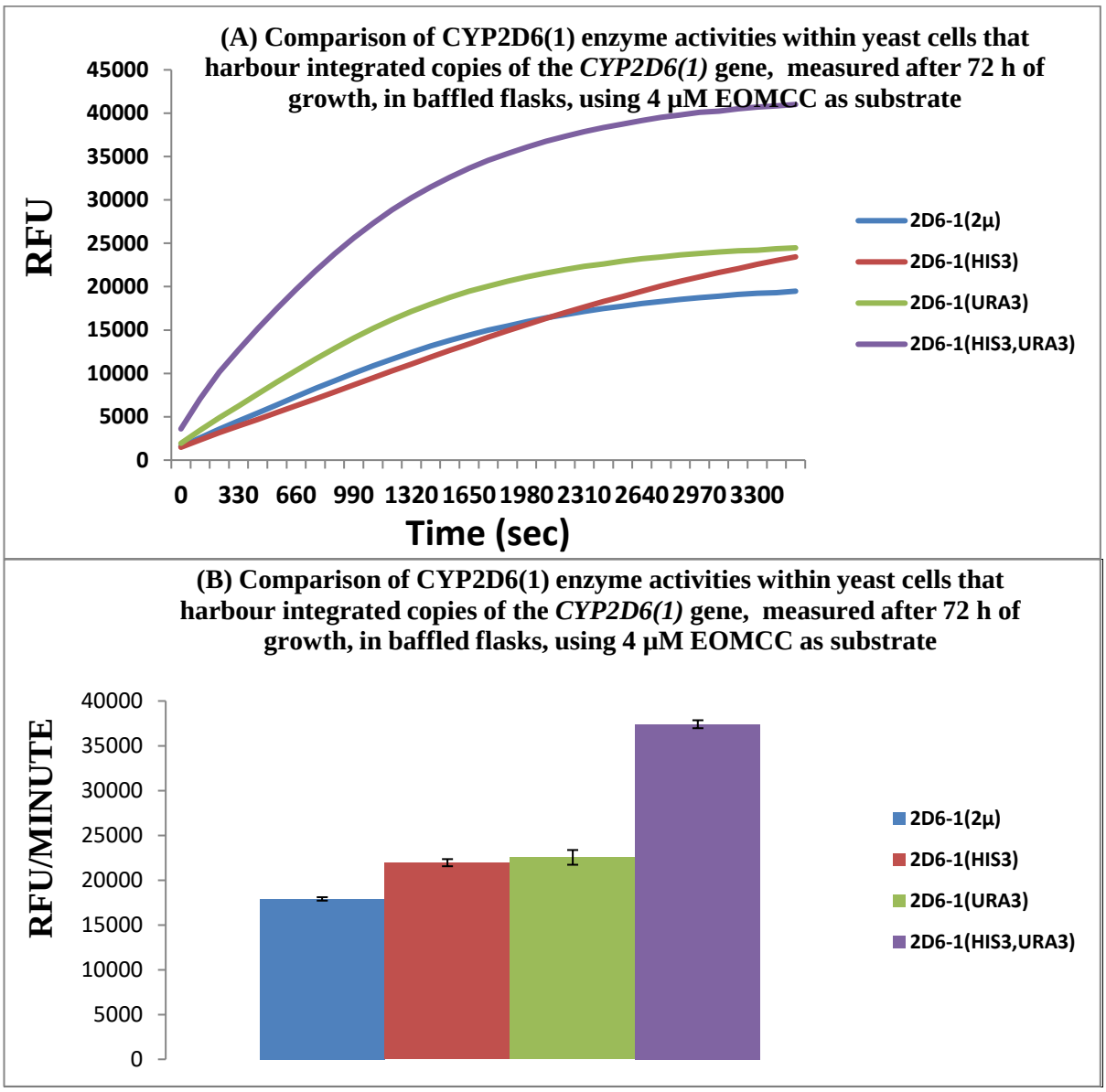


Figure 5.14. The graph (A) shows the comparison of CYP2D6(1) enzyme activities within cells of different yeast strains, derived from YY7. They contain the CYP2D6(1) gene in 1 integrated copy (two strains), 2 integrated copies (one strain), and the CYP2D6(1) gene encoded by an episomal plasmid. The graphs (A) represent the average of results obtained from three

independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

Figure 5.14 depicts the expression of human CYP2D6(1) enzyme in baker's yeast. The results show that the 2-copy integrated strain YY7::2D6-1(HIS3,URA3), referred to as 2D6-1(HIS3,URA3), has the highest activity amongst the four strains. This particular strain was used to prepare CYP2D6(1) (Val^{374}) microsomal enzyme.

5.3.10 Comparison of amounts of CYP2D6(1) (Val^{374}) microsomal enzyme isolated from yeast strains containing CYP2D6(1) gene which is (a) chromosomally integrated at two different loci and (b) encoded by an episomal plasmid

In order to evaluate the true expression levels of CYP2D6(1) enzyme in the strain YY7::2D6-1(HIS3,URA3), referred to as 2D6-1(HIS3,URA3) in Figure 5.15, microsomal CYP2D6(1) enzyme was isolated. Both strains were grown in 400 ml of YPD cell culture, under identical conditions. The correctly folded CYP protein, which provides the activity, was determined at 450 nm via CO difference spectroscopy (see Chapter 2, Section 2.8.3.1). Results shown in panels (A) and (B) of Figure 15 clearly show that the amount of enzyme produced by this integrated strain is more than the enzyme obtained from the episomal plasmid containing strain YY7::2D6-1(2μ), referred to as 2D6(2μ) in Figure 5.15.

Panel C (Figure 5.15) shows that the strain containing 2 integrated copies of the *CYP2D6(1)* gene produced 59 nanomoles of CYP2D6(1) (Val^{374}) enzyme while the episomal produced only 15 nanomoles from 400 ml cell cultures. Panel D depicts a

Western blot of CYP2D6(1) proteins obtained from two different strains. One can plainly see that the strain which expresses 2 integrated copies of human *CYP2D6(1)* gene produce more protein than the strain that expresses *CYP2D6(1)* from an episomal plasmid.

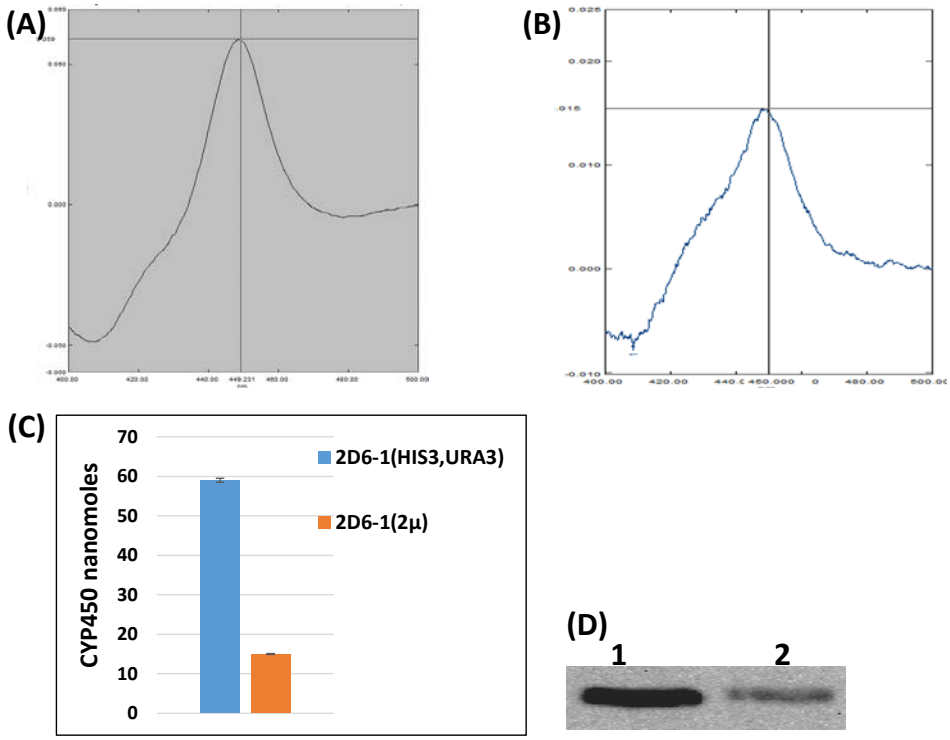


Figure 5.15. (A), (B): P450 contents, in the strains YY7::2D6-1(HIS3,URA3) [2D6-1(HIS3,URA3); (A)] and YY7::2D6-1(2µ) [2D6-1(2µ); (B)] were determined via CO difference spectroscopy using 1.0 µg of microsomal protein. (C) The amount of CYP450 (in nanomoles), obtained from the two strains (from 400 ml of YPD culture grown in baffled flasks), and represented as bar plots. The data represent mean ± S.D. of three independent experiments. (D) Western blots where 10 µg of total protein from the strains YY7::2D6-1(HIS3,URA3) (lane 1), and YY7::2D6-1(2µ) (lane 2) were probed with a CYP2D6 specific antibody (Santa Cruz Biotechnology, Cat no:sc130366).

5.3.11 Comparison of human CYP2D6(2) (Met^{374}) enzyme activities, expressed within yeast cells, from strains that contain 1 and 2 integrated copies of CYP2D6(2) gene

To find a yeast strain that would produce high amounts of human CYP2D6(2) enzyme [CYP2D6(Met^{374}); see Chapter 4, Section4.6.5], four new strains were created from the strain YY7 that contains only *ΔhRDM* at the *LEU2* locus.

YY7 cells were transformed with an

(1) Episomal (pSYE263/h_CYP2D6(2)_yc; Figure 3.38 , Chapter 3) to create a control strain,

(2) Integrating plasmid (YIpHisADH2S/CYP2D6(2)_yc; Figure 4.48, Chapter 4),

(3) Integrating plasmid (YIpUraADH2S/CYP2D6(2)_yc; Figure 4.50, Chapter 4).

The resultant strains obtained after transformation were named

(i) YY7::2D6-2(2μ), the control strain, [referred to as 2D6-2(2μ) in Figure 5.16],

(ii) YY7::2D6-2(HIS3) [referred to as 2D6-2(HIS3) in Figure 5.16], and

(iii) YY7::2D6-2(URA3) [referred to as 2D6-2(URA3) in Figure 5.16].

A second copy of the human *CYP2D6(2)* gene was introduced at the *URA3* locus, via transformation, into the strain YY7::2D6-2(HIS3). The resultant strain was named YY7::2D6-2(HIS3,URA3), referred to as 2D6-2(HIS3,URA3) in Figure 5.16.

The yeast strains were grown in YPD medium for 72 h, replenished every 24 h with new medium. The CYP2D6-2 [i.e. CYP2D6(Met^{374})] enzyme activities, within cells, were

monitored in (1X 10^7) cells harvested after 72 h of growth. Each culture was grown identically in YPD medium with an initial inoculum of equal number of cells. Fresh YPD medium was provided at each 24 h cycle of growth. The results in Figure 5.16 show the CYP2D6(2) (Met^{374}) enzyme activities obtained from four different yeast strains. The CYP2D6 activity of YY7::2D6-1(HIS3,URA3) (Val^{374}) was included for the sake of comparison, in panels (A) and (B) of Figure 5.16. The results show that the amounts produced by the strain YY7::2D6-1(HIS3,URA3) that produces the Val^{374} variant are marginally more than the amounts from the strain YY7::2D6-2(HIS3,URA3) that produces CYP2D6(Met^{374}).

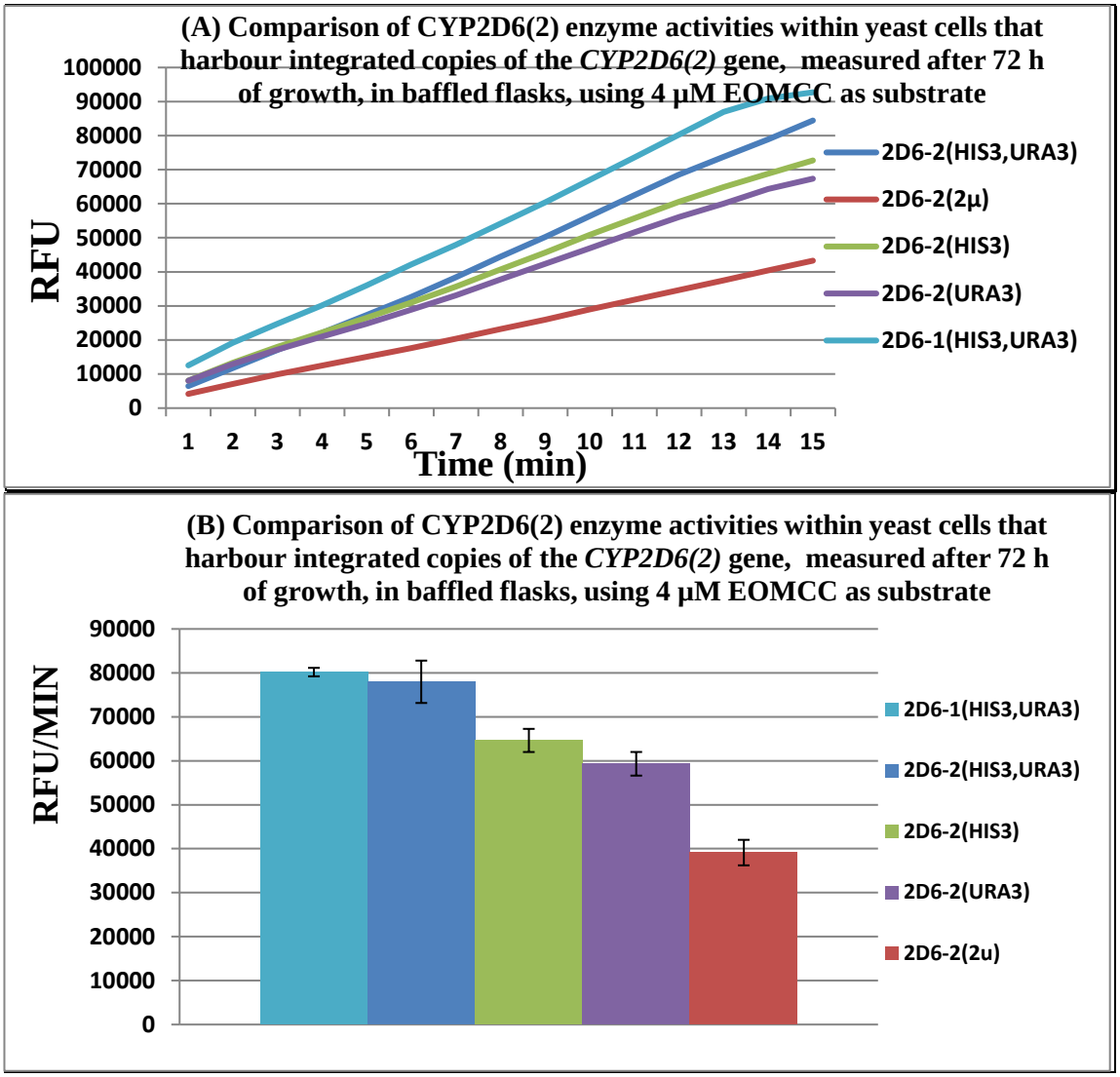


Figure 5.16. The graph (A) shows the comparison of CYP2D6(2) enzyme activities within cells of different yeast strains, derived from YY7. They contain the CYP2D6(2) gene in 1 integrated copy (two strains), 2 integrated copies (one strain), and the CYP2D6(2) gene encoded by an episomal plasmid. The graphs (A) represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

Figure 5.16 depicts the expression of human CYP2D6(2) enzyme in baker's yeast. The results show again that the 2-copy integrated strain YY7::2D6-2(HIS3,URA3), referred to as 2D6-2(HIS3,URA3), has the highest activity amongst the four 2D6-2 strains. This particular strain was used to prepare CYP2D6(2) (Met^{374}) microsomal enzyme.

5.3.12 Comparison of amounts of CYP2D6(2) (Met^{374}) microsomal enzyme isolated from strains containing CYP2D6(2) gene which is (a) chromosomally integrated at two different loci and (b) encoded by an episomal plasmid

With the aim of evaluating the true expression levels of CYP2D6(2) enzyme in the strain YY7::2D6-2(HIS3,URA3), referred to as 2D6-2(HIS3,URA3) in Figure 5.17, microsomal CYP2D6(2) enzyme was isolated. As in other instances, both strains were grown in 400 ml of YPD cell culture, under exactly the same conditions as before. The correctly folded CYP protein, which provides the activity, was determined at 450 nm via CO difference spectroscopy (see Chapter 2, Section 2.8.3.1). Results shown in panels (A) and (B) of Figure 5.17 clearly show that the amount of enzyme produced by this integrated strain is more than the enzyme obtained from the episomal plasmid containing strain YY7::2D6-2(2µ), referred to as 2D6(2µ) in Figure 5.17.

Panel C (Figure 5.17) shows that the strain containing 2 integrated copies of the *CYP2D6(2)* gene produced 52 nanomoles of CYP2D6(1) (Met^{374}) enzyme while the episomal produced only 9 nanomoles from 400 ml cell cultures. Panel D depicts a Western blot of CYP2D6(2) proteins obtained from three different strains. One can plainly see that the two strains which expresses 1 integrated copy and 2 integrated copies of human *CYP2D6(2)* gene produce more CYP protein than the strain that expresses *CYP2D6(2)* from an episomal plasmid.

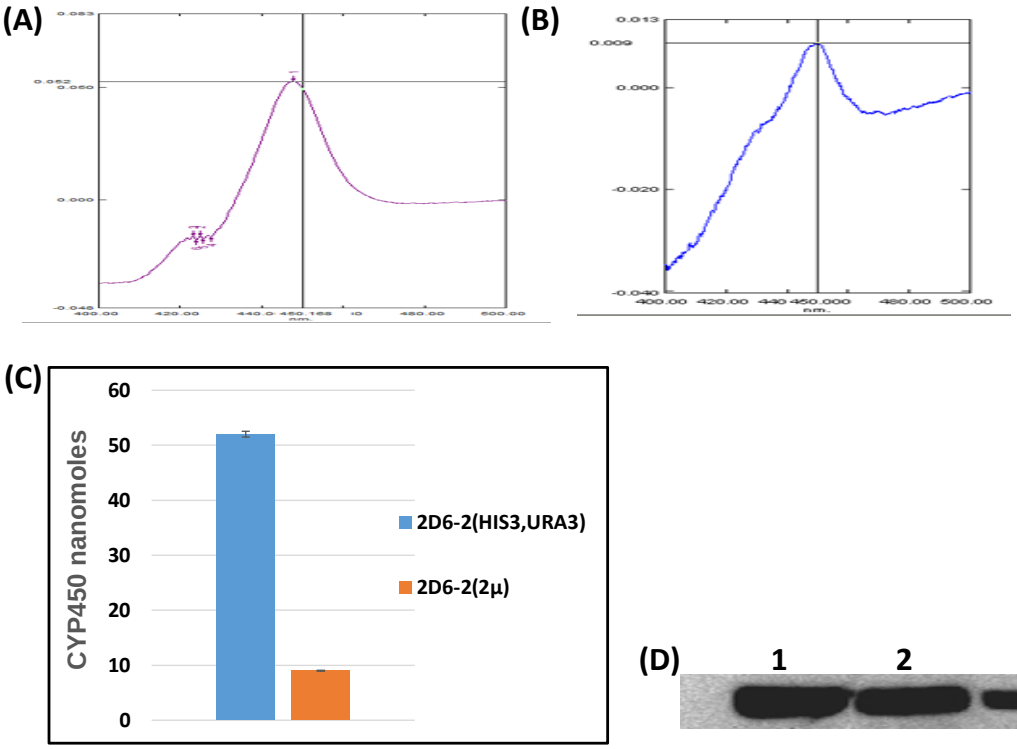


Figure 5.17. (A), (B): P450 contents, in the strains YY7::2D6-2(HIS3,URA3) [2D6-2(HIS3,URA3); (A)] and YY7::2D6-2(2µ) [2D6-1(2µ); (B)] were determined via CO difference spectroscopy using 1.0 µg of microsomal protein. (C) The amount of CYP450 (in nanomoles), obtained from the two strains (from 400 ml of YPD culture grown in baffled flasks), and represented as bar plots. The data represent mean ± S.D. of three independent experiments. (D) Western blots where 10 µg of total protein from the strains YY7::2D6-2(HIS3,URA3) (lane 1), YY7::2D6-2(HIS3) (lane 2),and YY7::2D6-1(2µ) (lane 3) were probed with a CYP2D6 specific antibody (Santa Cruz Biotechnology, Cat no:sc130366).

5.3.13 Comparison of human CYP2C19 enzyme activities, expressed within yeast cells, from strains that contain 1 and 2 integrated copies of CYP2C19 gene

With the aim of identifying a yeast strain that would produce high amounts of human CYP2C19 enzyme, four new strains were created from the strain YAB79 that contains *ΔhRDM* and cytochrome *b5* genes at the *LEU2* and *TRP1* loci, respectively.

YAB79 cells were transformed with an

(1) Episomal (pSYE263/h_CYP2C19_yc; Figure 3.17, Chapter 3) to create a control strain,

(2) Integrating plasmid (YIpHisADH2S/CYP2C19_yc; Figure 4.53, Chapter 4),

(3) Integrating plasmid (YIpUraADH2S/CYP2C19_yc; Figure 4.55, Chapter 4).

The resultant strains obtained after transformation were named

(i) YAB79::2C19(2µ), the control strain, [referred to as 2C19(2µ) in Figure 5.18],

(ii) YAB79::2C19(HIS3) [referred to as 2C19(HIS3) in Figure 5.18], and

(iii) YAB79::2C19(URA3) [referred to as 2C19(URA3) in Figure 5.18].

The strain YAB79::2C19(HIS3) was used to introduce a second copy of the human *CYP2C19* gene at the *URA3* locus. The resultant strain obtained after transformation was named YAB79::2C19(HIS3,URA3), referred to as 2C19(HIS3,URA3) in Figure 5.18.

The yeast strains were grown in YPD medium for 72 h, replenished with new medium every 24 h. The CYP2C19 activities, within cells, were monitored in (1X 10^7) cells

harvested after 72 h of growth. Each culture was started with an inoculum of equal number of cells. All cell cultures were grown identically, with fresh YPD medium being provided at the same time points of growth. The results in Figure 5.18 show comparative CYP2C19 enzyme activities obtained from four different strains.

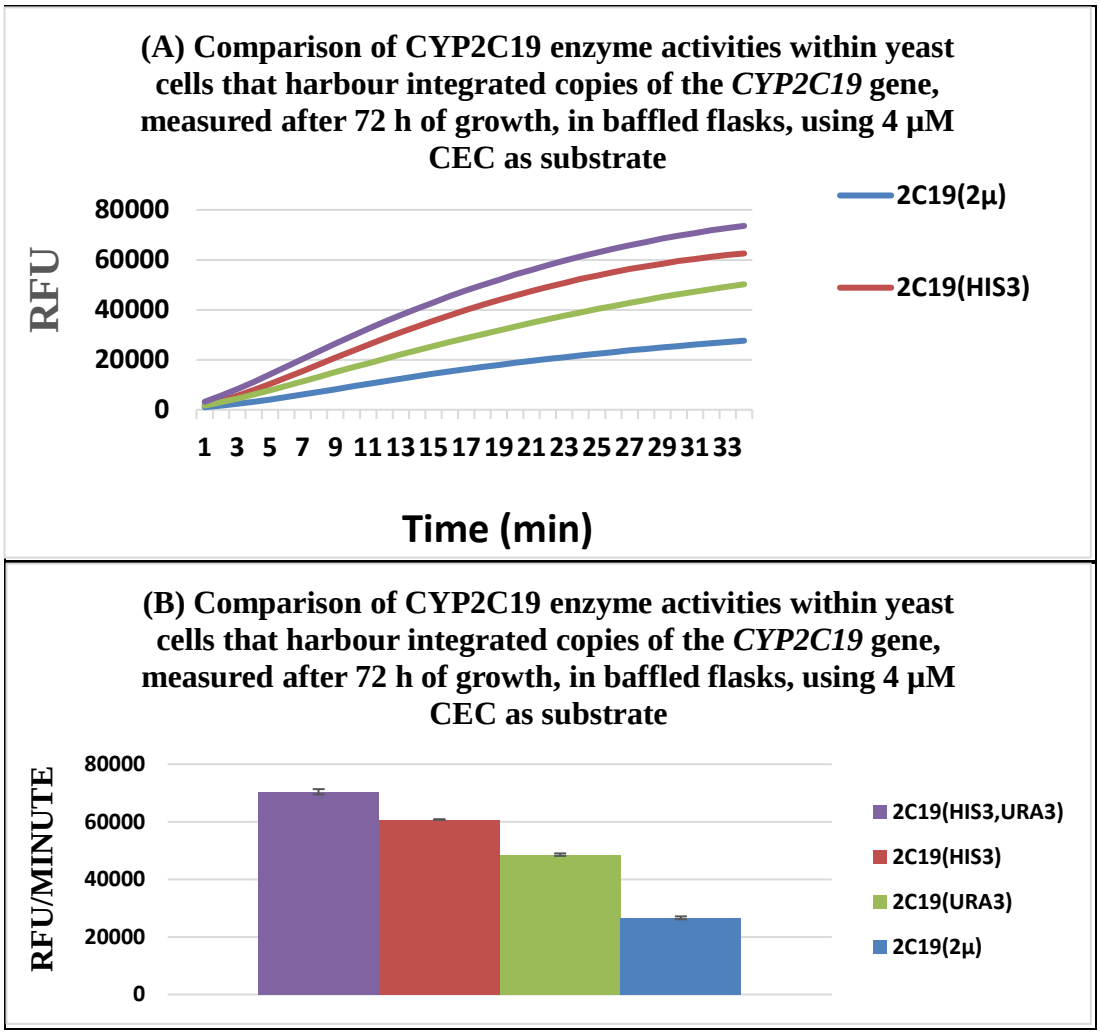


Figure 5.18. The graph (A) shows the comparison of CYP2C19 enzyme activities within cells of different yeast strains, derived from YAB79. They contain the CYP2C19 gene in 1 integrated copy, 2 integrated copies, and the CYP2C19 gene encoded by an episomal plasmid. The graphs (A) represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

Figure 18 depicts the expression of human CYP2C19 in baker's yeast. The results show that the 2-copy integrated strain YAB79::2C19(HIS3,URA3), referred to as 2C19(HIS3,URA3), has the highest activity. This strain was used to prepare CYP2C19 microsomal enzyme.

5.3.14 Comparison of amounts of CYP2C19 microsomal enzyme isolated from yeast strains containing CYP2C19 gene which is (a) chromosomally integrated at two different loci and (b) encoded by an episomal plasmid

In order to evaluate the true expression levels of CYP2C19 enzyme in the strain YAB79::2C19(HIS3,URA3), referred to as 2C19(HIS3,URA3) in Figure 5.18, microsomal CYP2C19 enzyme was isolated. Both strains were grown in 400 ml of YPD cell culture, under exactly the same conditions. The correctly folded CYP protein, which provides the activity, was determined at 450 nm via CO difference spectroscopy (see Chapter 2, Section 2.8.3.1). Results shown in panels (A) and (B) of Figure 5.19 clearly show that the amount of enzyme produced by this integrated strain is more than the enzyme obtained from the episomal plasmid containing strain YAB79::2C19(2µ), referred to as 2C19(2µ) in Figure 5.19. Comparison of the graphs in panels (A) and (B) reveals that the amounts of cytochrome b5 expressed is much less in YAB79::2C19(HIS3,URA3) cells than in the strain YAB79::2C19(2µ). As has been shown later in this Chapter, after isolation of the microsomal enzyme, cytochrome b5 levels may not be at all relevant for CYP450 activity.

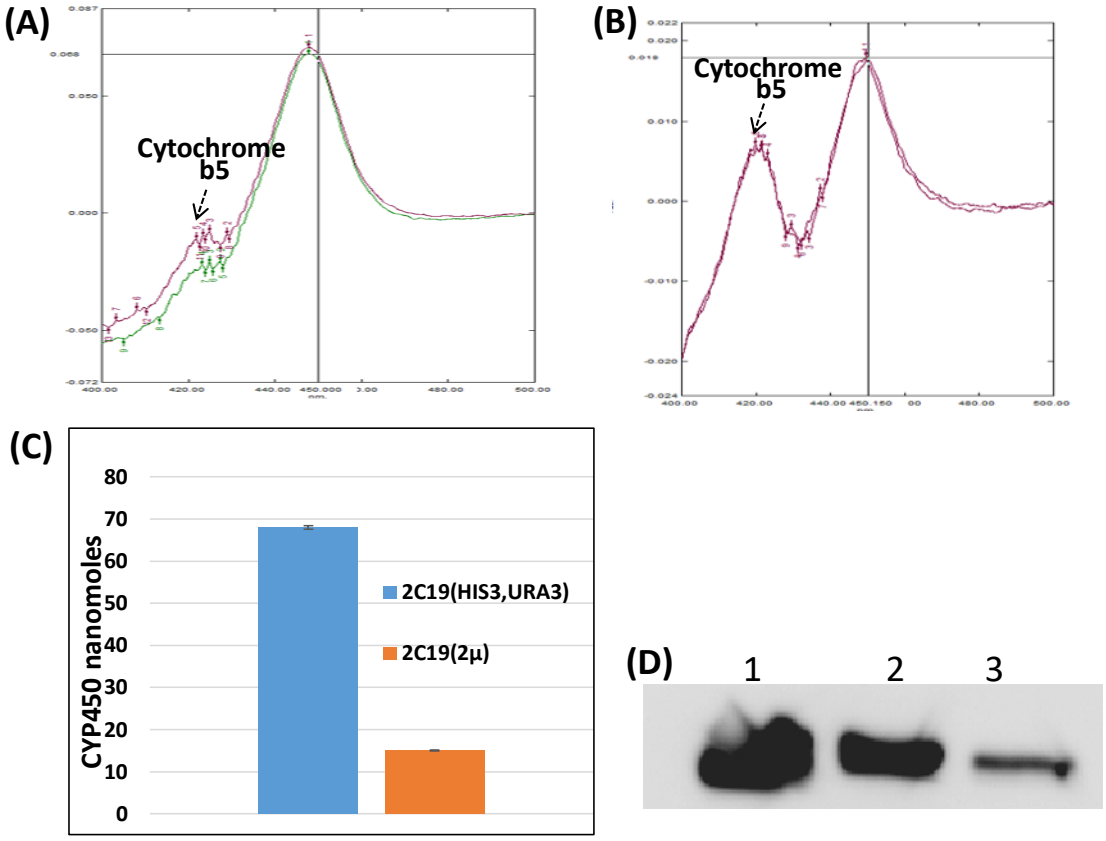


Figure 5.19. (A), (B): P450 contents, in the strains YAB79::2C19(HIS3,URA3) [2C19(HIS3,URA3); (A)] and YAB79::2C19(2µ) [2C19(2µ); (B)] were determined by CO difference spectroscopy using 1.0 µg of microsomal protein. (C) The amount of CYP450 (in nanomoles), obtained from the 2 strains (from 400 ml of YPD culture grown in baffled flasks), and represented as bar plots. The data represent mean ± S.D. of three independent experiments. (D) Western blots where 10 µg of total protein from the strains YAB79::2C19(HIS3,URA3) (lane 1), YAB79::2C19(HIS3) (lane 2) and YAB79::2C19(2µ) (lane 3) were probed with a CYP2C19 specific antibody (Santa Cruz Bitechnology, Cat no:sc25581).

Panel C (Figure 19) shows that the strain containing 2 integrated copies of the CYP2C19 gene produced 68 nanomoles of *CYP2C19* enzyme while the episomal produced only 15 nanomoles from 400 ml of cell culture. Panel D depicts a Western blot of CYP2C19 proteins obtained from different strains. One can again see clearly that the strains which express 1-2 integrated copies of human *CYP2C19* gene produce more protein than the strain that expresses *CYP2C19* from an episomal plasmid.

5.3.15 Comparison of human CYP2C9 enzyme activities, expressed within yeast cells, from strains that contain 1 and 2 integrated copies of CYP2C9 gene

With the aim of identifying a yeast strain that would produce high amounts of human CYP2C9 enzyme, four new strains were created from the strain YAB79 that contains *ΔhRDM* and cytochrome *b5* genes at the *LEU2* and *TRP1* loci, respectively.

YAB79 cells were transformed with an

(1) Episomal (pSYE263/h_CYP2C9_yc; Figure 3.12, Chapter 3) to create a control strain,

(2) Integrating plasmid (YIpHisADH2S/CYP2C9_yc; Figure 4.60, Chapter 4),

(3) Integrating plasmid (YIpUraADH2S/CYP2C9_yc; Figure 4.58, Chapter 4).

The resultant strains obtained after transformation were named

(i) YAB79::2C9(2µ), the control strain, [referred to as 2C9(2µ) in Figure 5.20],

(ii) YAB79::2C9(HIS3) [referred to as 2C9(HIS3) in Figure 5.20], and

(iii) YAB79::2C9(URA3) [referred to as 2C9(URA3) in Figure 5.20].

The strain YAB79::2C9(HIS3) was used to introduce a second copy of the human *CYP2C9* gene at the *URA3* locus. The resultant strain obtained after transformation was named YAB79::2C9(HIS3,URA3), referred to as 2C9(HIS3,URA3) in Figure 5.20.

The yeast strains were grown in YPD medium for 72 h, replenished with new medium every 24 h. The CYP2C9 activities, within cells, were monitored in ($1X\ 10^7$) cells

harvested after 72 h of growth. Each culture was started with an inoculum of equal number of cells. All cell cultures were grown identically, with fresh YPD medium being provided at the same time points of growth. The results in Figure 5.20 show comparative CYP2C9 enzyme activities obtained from four different strains.

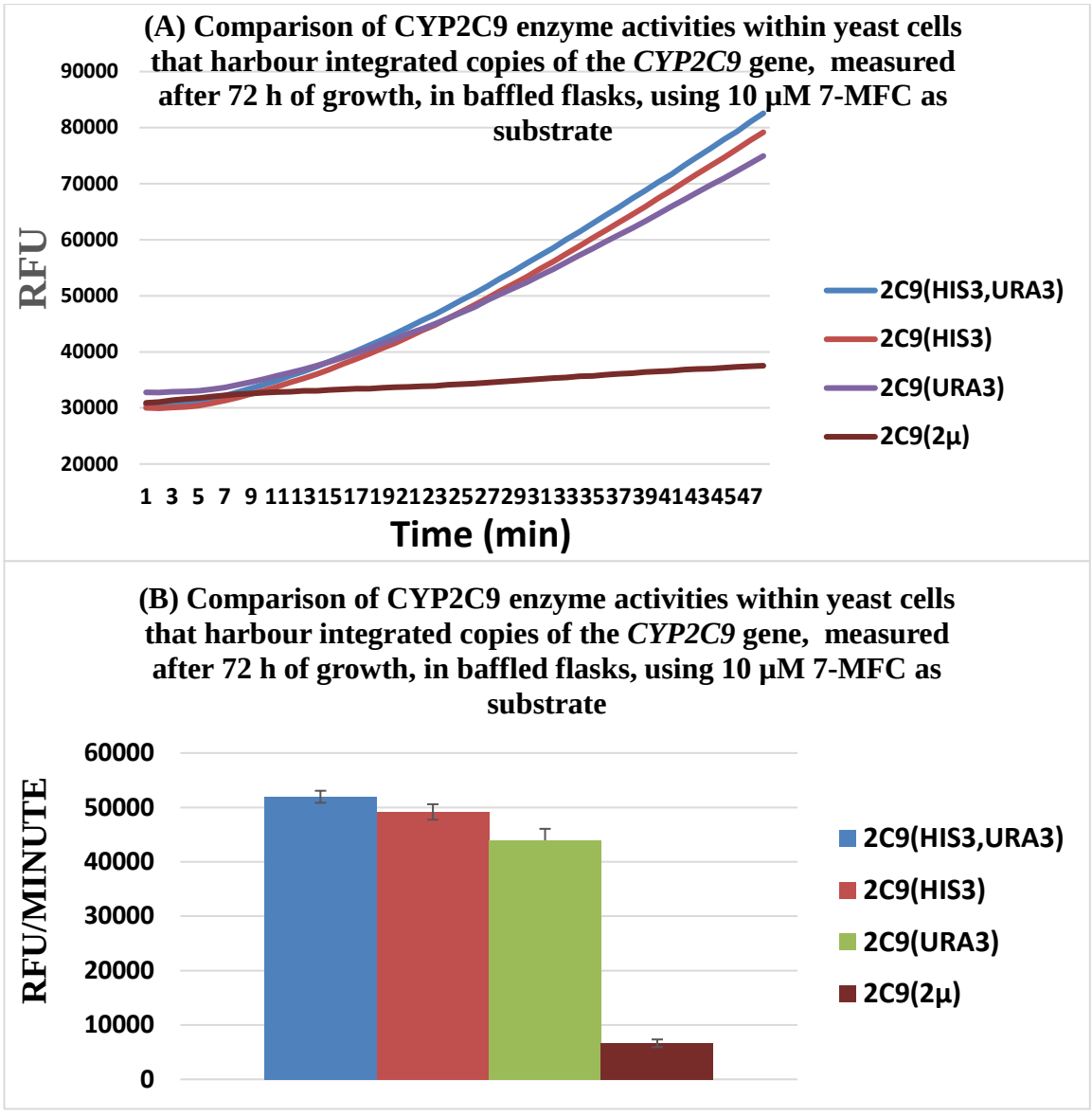


Figure 5.20. The graph (A) shows the comparison of CYP2C9 enzyme activities within cells of different yeast strains, derived from YAB79. They contain the CYP2C9 gene in 1 integrated copy, 2 integrated copies, and the CYP2C9 gene encoded by an episomal plasmid. The graphs (A) represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

Figure 20 depicts the expression of human CYP2C9 enzyme in baker's yeast. The results show that the 2-copy integrated strain YAB79::2C9(HIS3,URA3), referred to as 2C19(HIS3,URA3), has slightly more activity than the 1-copy strains. The 2-copy strain was used to prepare CYP2C9 microsomal enzyme.

5.3.16 Comparison of amounts of CYP2C9 microsomal enzyme isolated from yeast strains containing CYP2C9 gene which is (a) chromosomally integrated at two different loci and (b) encoded by an episomal plasmid

In order to evaluate the true expression levels of CYP2C9 enzyme in the strain YAB79::2C9(HIS3,URA3), referred to as 2C9(HIS3,URA3) in Figure 5.20, microsomal CYP2C9 enzyme was isolated. Both strains were grown in 400 ml of YPD cell culture, under exactly the same conditions. The correctly folded CYP protein, which provides the activity, was determined at 450 nm via CO difference spectroscopy (see Chapter 2, Section 2.8.3.1). Results shown in panels (A) and (B) of Figure 5.21 clearly show that the amount of enzyme produced by this integrated strain is more than the enzyme obtained from the episomal plasmid containing strain YAB79::2C9(2μ), referred to as 2C9(2μ) in Figure 5.21.

Panel C (Figure 5.21) shows that the strain containing 2 integrated copies of the CYP2C9 gene produced 206 nanomoles of *CYP2C9* enzyme while the episomal produced only 10 nanomoles from 400 ml of cell culture. Panel D depicts a Western blot of CYP2C9 proteins obtained from different strains. One can see that the strains which express 1-2

integrated copies of human *CYP2C9* gene produce more protein than the strain that expresses *CYP2C9* from an episomal plasmid.

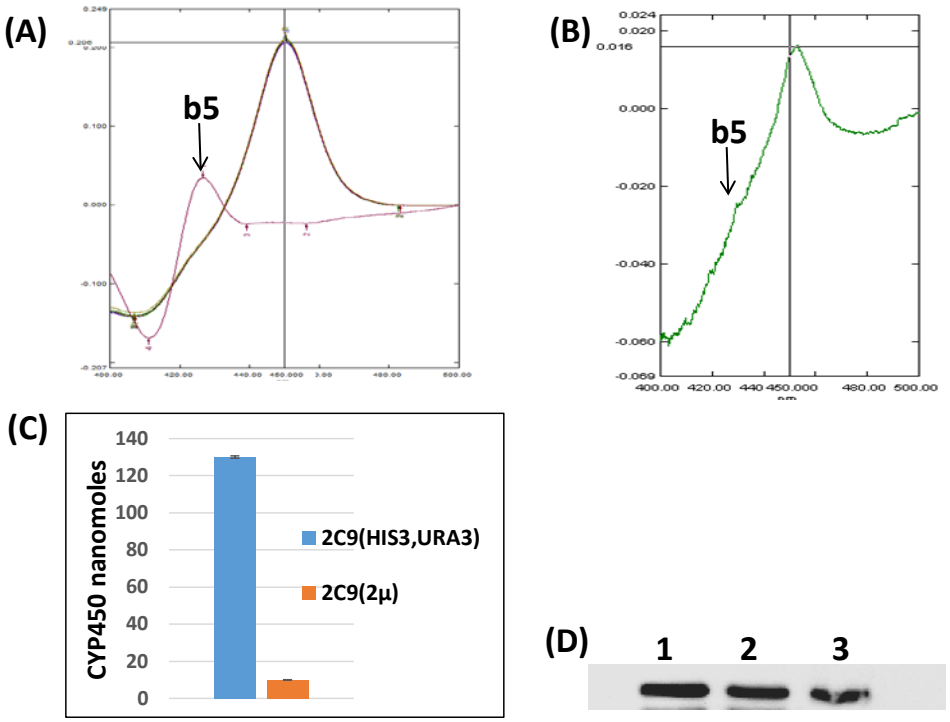


Figure 5.21. (A), (B): P450 contents, in the strains YAB79::2C9(HIS3,URA3) [2C9(HIS3,URA3); (A)] and YAB79::2C9(2µ) [2C9(2µ); (B)] were determined by CO difference spectroscopy using 1.0 µg of microsomal protein. (C) The amount of CYP450 (in nanomoles), obtained from the 2 strains (from 400 ml of YPD culture grown in baffled flasks), and represented as bar plots. The data represent mean ± S.D. of three independent experiments. (D) Western blots where 10 µg of total protein from the strains YAB79::2C9(HIS3,URA3) (lane 1), YAB79::2C9(HIS3) (lane 2) and YAB79::2C9(2µ) (lane 3) were probed with a CYP2C9 specific antibody (Santa Cruz Biotechnology, Cat no:sc-374421).

5.3.17 Comparison of human CYP1B1 enzyme activities, expressed within yeast cells, from strains that contain 1 and 2 integrated copies of CYP1B1 gene

The aim was again to identify a yeast strain that would produce high amounts of human CYP1B1 enzyme. Four new strains were created from the strain YY7 that contains only *ΔhRDM* at the *LEU2* locus.

YY7 cells were transformed with an

(1) Episomal (pSYE263/h_CYP1B1_yc; Figure 3.67, Chapter 3) to create a control strain,

(2) Integrating plasmid (YIpHisADH2S/CYP1B1_yc; Figure 4.72, Chapter4),

(3) Integrating plasmid (YIpUraADH2S/CYP1B1_yc; Figure 4.74, Chapter 4).

The resultant strains obtained after transformation were named

(i) YY7::1B1(2μ), the control strain, [referred to as 1B1(2μ) in Figure 5.22],

(ii) YY7::1B1(HIS3) [referred to as 1B1(HIS3) in Figure 5.22], and

(iii) YY7::1B1(URA3) [referred to as 1B1(URA3) in Figure 5.22].

The strain YY7::1B1(HIS3) was used to introduce a second copy of the human *CYP1B1* gene at the *URA3* locus. The resultant strain obtained after transformation was named YY7::1B1(HIS3,URA3), referred to as 1B1(HIS3,URA3) in Figure 5.22.

The yeast strains were grown in YPD medium for 72 h, replenished with new medium every 24 h. The CYP1B1 activities, within cells, were monitored in (1X 10^7) cells

harvested after 72 h of growth. Each culture was started with an inoculum of equal number of cells. All cell cultures were grown identically, with fresh YPD medium being provided at the same time points of growth. The results in Figure 5.22 show comparative CYP1B1 enzyme activities obtained from four different strains.

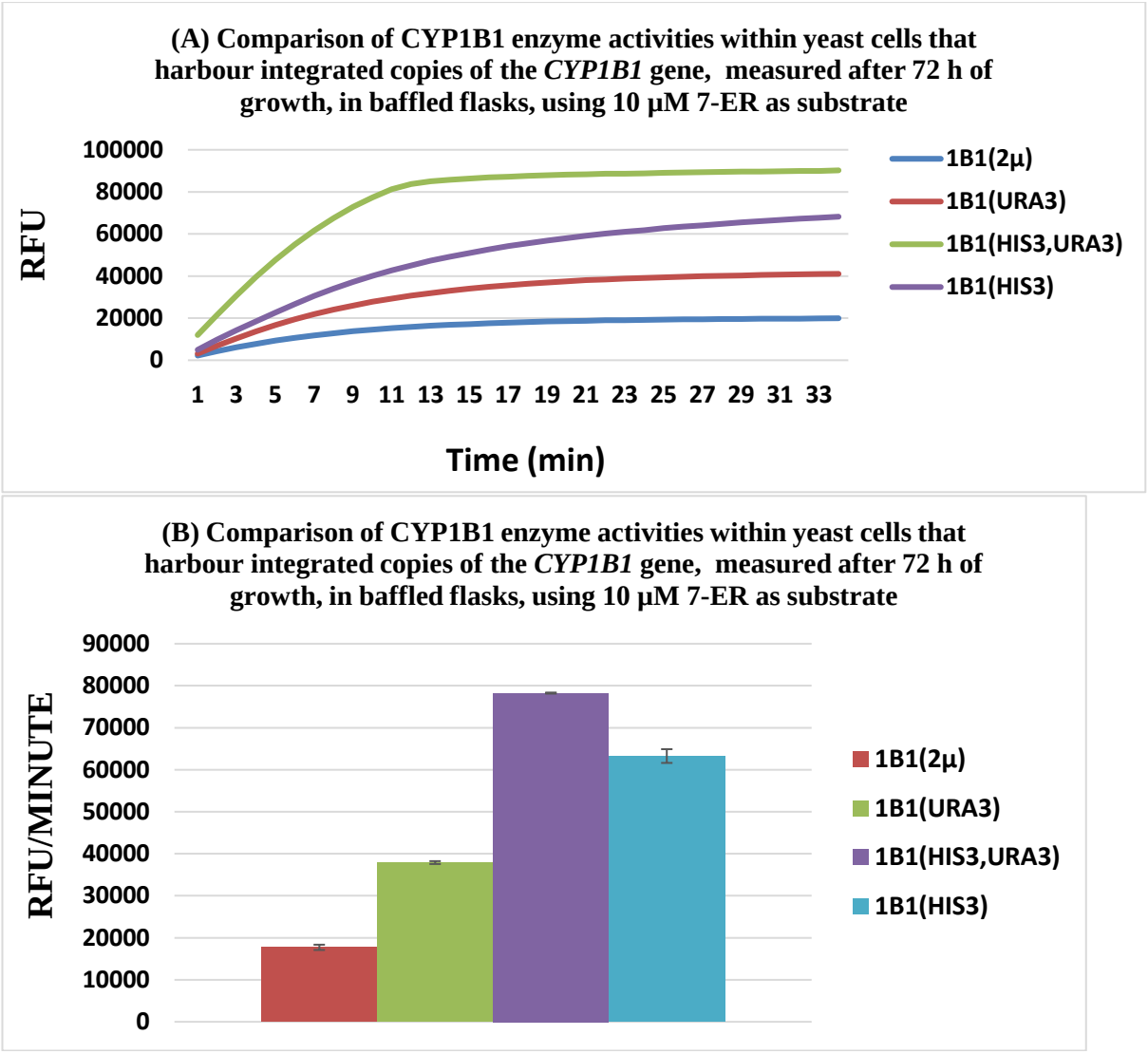


Figure 5.22. The graph (A) shows the comparison of CYP1B1 enzyme activities within cells of different yeast strains, derived from YY7. They contain the CYP1B1 gene in 1 integrated copy, 2 integrated copies, and the CYP1B1 gene encoded by an episomal plasmid. The graphs (A) represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

Figure 5.22 depicts the expression of human CYP1B1 enzyme in baker's yeast. The results show that the 2-copy integrated strain YY7::1B1(HIS3,URA3), referred to as 1B1(HIS3,URA3), has slightly more activity than the 1-copy strain which expresses enzyme from the *HIS3* locus. The 2-copy strain was used to prepare CYP1B1 microsomal enzyme.

5.3.16 Comparison of amounts of CYP1B1 microsomal enzyme isolated from yeast strains containing CYP1B1 gene which is (a) chromosomally integrated at two different loci and (b) encoded by an episomal plasmid

In order to evaluate the true expression levels of CYP1B1 enzyme in the strain YY7::1B1(HIS3,URA3), referred to as 1B1(HIS3,URA3) in Figure 5.22, microsomal CYP1B1 enzyme was isolated. Both strains were grown in 400 ml of YPD cell culture, under exactly the same conditions. The correctly folded CYP protein, which provides the activity, was determined at 450 nm via CO difference spectroscopy (see Chapter 2, Section 2.8.3.1). Results shown in panels (A) and (B) of Figure 23 clearly show that the amount of enzyme produced by this integrated strain is more than the enzyme obtained from the episomal plasmid containing strain YY7::1B1(2µ), referred to as 1B1(2µ) in Figure 5.23.

Panel C (Figure 5.23) shows that the strain containing 2 integrated copies of the CYP1B1 gene produced 105 nanomoles of *CYP1B1* enzyme while the episomal produced 10 nanomoles from 400 ml of cell culture. Panel D depicts a Western blot of CYP1B1 proteins obtained from different strains. One can see that the strains which express 1-2

integrated copies of human *CYP1B1* gene clearly produce more protein than the strain that expresses *CYP1B1* from an episomal plasmid.

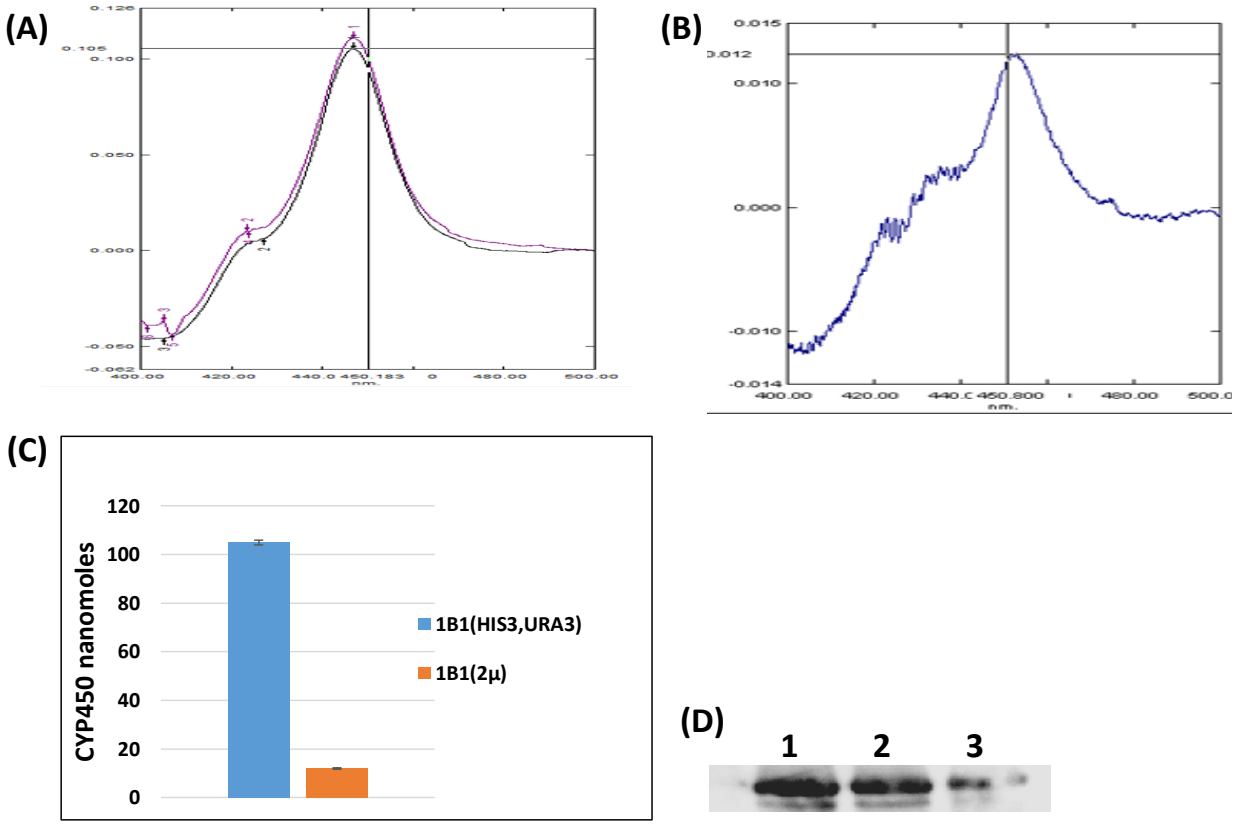


Figure 5.23. (A), (B): P450 contents, in the strains YY7::1B1(HIS3,URA3) [1B1(HIS3,URA3); (A)] and YY7::1B1(2µ) [1B1(2µ); (B)] were determined by CO difference spectroscopy using 1.0 µg of microsomal protein. (C) The amount of CYP450 (in nanomoles), obtained from the 2 strains (from 400 ml of YPD culture grown in baffled flasks), and represented as bar plots. The data represent mean ± S.D. of three independent experiments. (D) Western blots where 10 µg of total protein from the strains YY7::1B1(HIS3,URA3) (lane 1), YY7::1B1(HIS3) (lane 2) and YY7::1B1(2µ) (lane 3) were probed with a CYP1B1 specific antibody (Santa Cruz Biotechnology, Cat no:sc-374228).

5.3.17 Comparison of human CYP1A1 enzyme activities, expressed within yeast cells, from strains that contain 1 and 2 integrated copies of CYP1A1 gene

The aim was again to identify a yeast strain that would produce high amounts of human CYP1A1 enzyme. Four new strains were created from the strain YY7 that contains only *ΔhRDM* at the *LEU2* locus.

YY7 cells were transformed with an

(1) Episomal (pSYE263/h_CYP1A1_yc; Figure 3.62, Chapter 3.62) to create a control strain,

(2) Integrating plasmid (YIpHisADH2S/CYP1A1_yc; Figure 4.67, Chapter4),

(3) Integrating plasmid (YIpUraADH2S/CYP1A1_yc; Figure 4.69, Chapter 4).

The resultant strains obtained after transformation were named

(i) YY7::1A1(2μ), the control strain, [referred to as 1A1(2μ) in Figure 5.24],

(ii) YY7::1A1(HIS3) [referred to as 1A1(HIS3) in Figure 5.24], and

(iii) YY7::1A1(URA3) [referred to as 1A1(URA3) in Figure 5.24].

The strain YY7::1A1(HIS3) was used to introduce a second copy of the human *CYP1A1* gene at the *URA3* locus. The resultant strain obtained after transformation was named YY7::1A1(HIS3,URA3), referred to as 1A1(HIS3,URA3) in Figure 5.24.

The yeast strains were grown in YPD medium for 72 h, replenished with new medium every 24 h. The CYP1A1 activities, within cells, were monitored in (1X 10^7) cells

harvested after 72 h of growth. Each culture was started with an inoculum of equal number of cells. All cell cultures were grown identically, with fresh YPD medium being provided at the same time points of growth. The results in Figure 5.24 show comparative CYP1A1 enzyme activities obtained from four different strains.

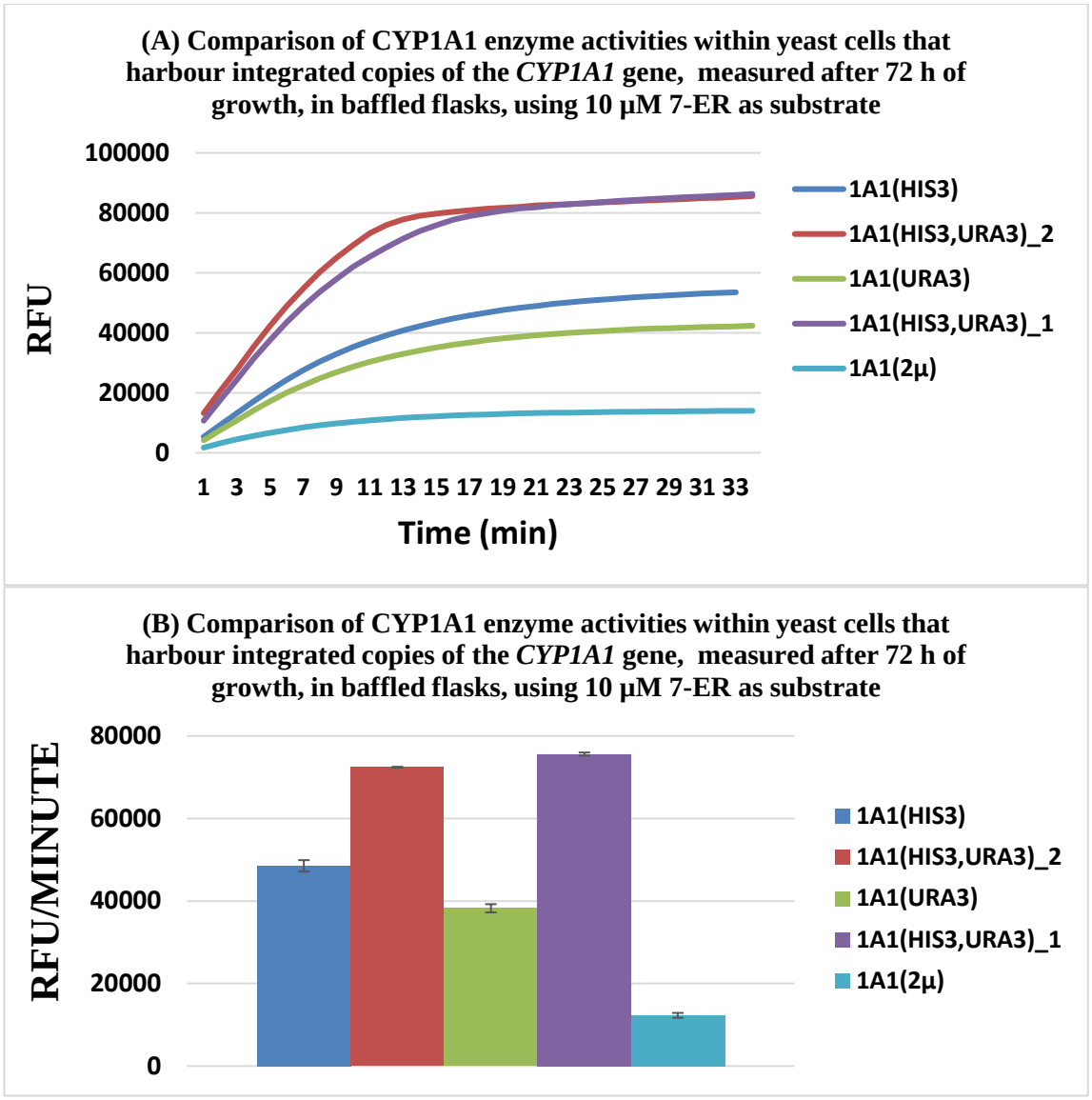


Figure 5.24. The graph (A) shows the comparison of CYP1A1 enzyme activities within cells of different yeast strains, derived from YY7. They contain the CYP1A1 gene in 1 integrated copy, 2 integrated copies, and the CYP1A1 gene encoded by an episomal plasmid. The graphs (A) represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

Figure 5.24 depicts the expression of human CYP1A1 enzyme in baker's yeast. The results show that the 2-copy integrated strain YY7::1A1(HIS3,URA3), referred to as 1A1(HIS3,URA3), has by far better activity than the 1-copy strains which express enzyme from the *HIS3* and *URA3* loci. The 2-copy strain was further used to prepare CYP1A1 microsomal enzyme.

5.3.18 Comparison of amounts of CYP1A1 microsomal enzyme isolated from yeast strains containing CYP1A1 gene which is (a) chromosomally integrated at two different loci and (b) encoded by an episomal plasmid

In order to evaluate the true expression levels of CYP1A1 enzyme in the strain YY7::1A1(HIS3,URA3), referred to as 1A1(HIS3,URA3) in Figure 5.24, microsomal CYP1A1 enzyme was isolated. Both strains were grown in 400 ml of YPD cell culture, under exactly the same conditions. The correctly folded CYP protein, which provides the activity, was determined at 450 nm via CO difference spectroscopy (see Chapter 2, Section 2.8.3.1). Results shown in panels (A) and (B) of Figure 5.25 clearly show that the amount of enzyme produced by this integrated strain is more than the enzyme obtained from the episomal plasmid containing strain YY7::1A1(2µ), referred to as 1A1(2µ) in Figure 5.25.

Panel C (Figure 5.25) shows that the strain containing 2 integrated copies of the *CYP1A1* gene produced 105 nanomoles of CYP1A1 enzyme while the episomal produced 10 nanomoles from 400 ml of cell culture. Panel D depicts a Western blot of CYP1A1 proteins obtained from different strains. One can see that the strains which express 1-2

integrated copies of human *CYP1A1* gene clearly produce more protein than the strain that expresses *CYP1A1* from an episomal plasmid.

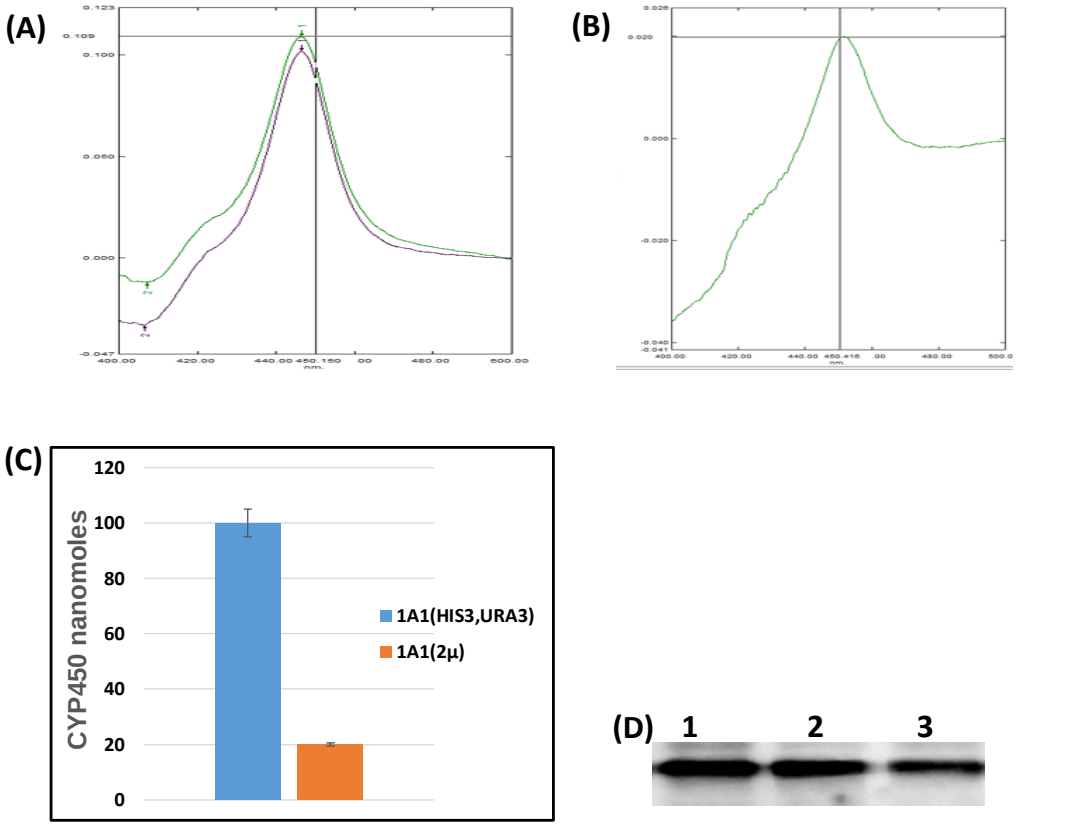


Figure 5.25. (A), (B): P450 contents, in the strains YY7::1A1(HIS3,URA3) [1A1(HIS3,URA3); (A)] and YY7:: 1A1(2µ) [1A1(2µ); (B)] were determined by CO difference spectroscopy using 1.0 µg of microsomal protein. (C) The amount of CYP450 (in nanomoles), obtained from the 2 strains (from 400 ml of YPD culture grown in baffled flasks), and represented as bar plots. The data represent mean ± S.D. of three independent experiments. (D) Western blots where 10 µg of total protein from the strains YY7::1A1(HIS3,URA3) (lane 1), YY7::1A1(HIS3) (lane 2) and YY7:: 1A1(2µ) (lane 3) were probed with a CYP1A1 specific antibody (Santa Cruz Biotechnology, Cat no:sc-393979).

5.3.19 Comparison of human CYP4F3A enzyme activities, expressed within yeast cells, from strains that contain 1 and 2 integrated copies of CYP4F3A gene

The aim was again to identify a yeast strain that would produce high amounts of human CYP4F3A enzyme. Four new strains were created from the strain YAB79 that contains *ΔhRDM* and cytochrome *b5* at the *LEU2* and *TRP1* loci, respectively.

YAB79 cells were transformed with an

(4) Episomal (pSYE263/h_CYP4F3A_yc; Figure 3.72, Chapter3) to create a control strain,

(5) Integrating plasmid (YIpHisADH2S/CYP4F3A_yc; Figure 4.62, Chapter 4),

(6) Integrating plasmid (YIpUraADH2S/CYP4F3A_yc; Figure 4.64, Chapter 4).

The resultant strains obtained after transformation were named

(iv) YAB79::4F3A(2µ), the control strain, [referred to as 4F3A(2µ) in Figure 26],

(v) YAB79::4F3A(HIS3) [referred to as 4F3A(HIS3) in Figure 5.26], and

(vi) YAB79::4F3A(URA3) [referred to as 4F3A(URA3) in Figure 5.26].

The strain YY7::4F3A(HIS3) was used to introduce a second copy of the human *CYP4F3A* gene at the *URA3* locus. The resultant strain obtained after transformation was named YY7::4F3A(HIS3,URA3), referred to as 4F3A(HIS3,URA3) in Figure 5.26.

The yeast strains were grown in YPD medium for 72 h, replenished with new medium every 24 h. The CYP4F3A activities, within cells, were monitored in (1X 10^7) cells

harvested after 72 h of growth. Each culture was started with an inoculum of equal number of cells. All cell cultures were grown identically, with fresh YPD medium being provided at the same time points of growth. The results in Figure 5.26 show comparative CYP4F3A enzyme activities obtained from four different strains.

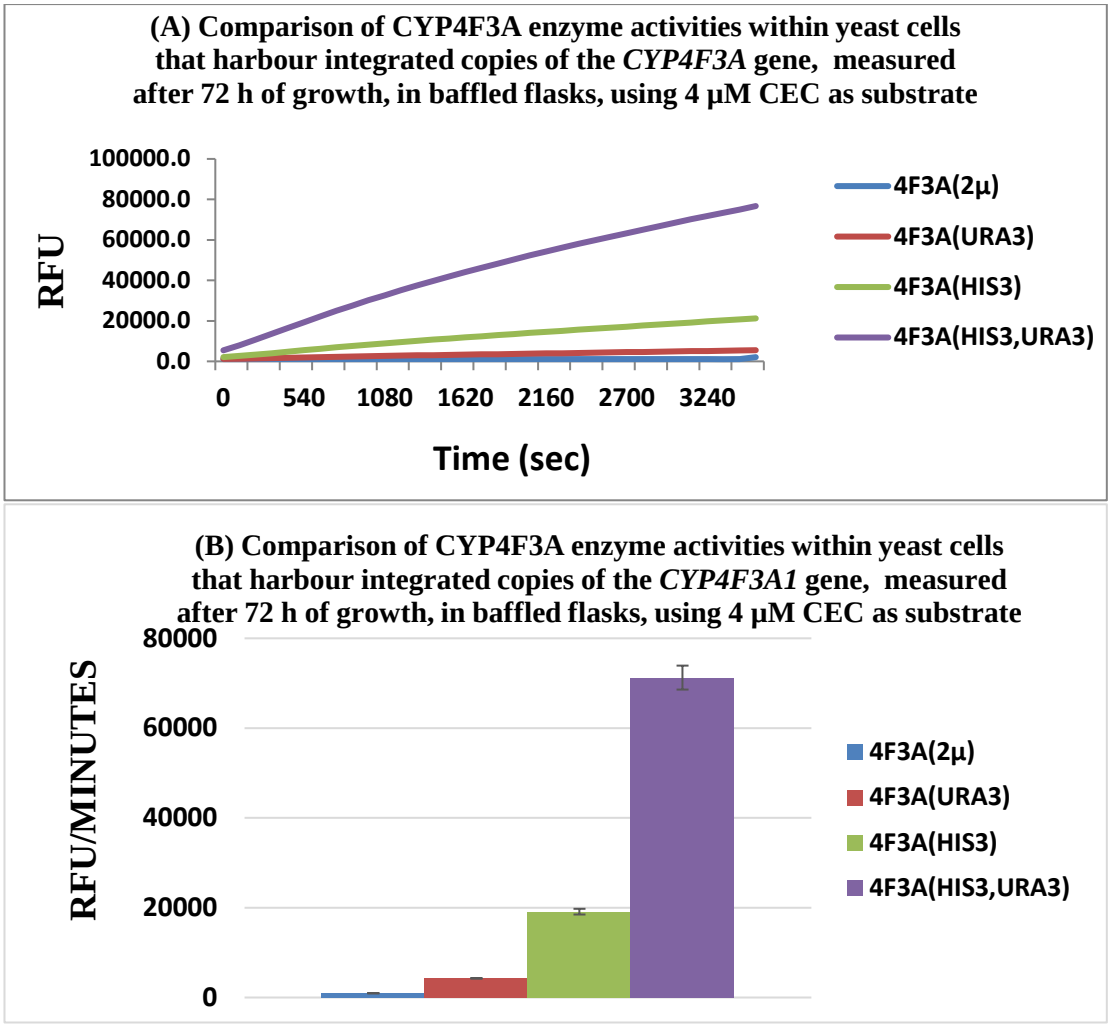


Figure 5.26. The graph (A) shows the comparison of CYP4F3A enzyme activities within cells of different yeast strains, derived from YAB79. They contain the CYP4F3A gene in 1 integrated copy, 2 integrated copies, and the CYP4F3A gene encoded by an episomal plasmid. The graphs (A) represent the average of results obtained from three independent experiments. The bar plot (B) mirrors the graphs in (A). The data represent mean ± S.D. of three independent experiments.

Figure 5.26 depicts the expression of human CYP4F3A enzyme in baker's yeast. The results show that the 2-copy integrated strain YAB79::4F3A(HIS3,URA3), referred to as 4F3A(HIS3,URA3), has by far the best activity compared to the other integrated strains. The strain [YAB79::4F3A(2µ)] containing the episomal plasmid hardly has any activity. The 2-copy strain was further used to prepare CYP4F3A microsomal enzyme from yeast cells.

5.3.20 Comparison of amounts of CYP4F3A microsomal enzyme isolated from yeast strains containing CYP4F3A gene which is (a) chromosomally integrated at two different loci and (b) encoded by an episomal plasmid

In order to evaluate the true expression levels of CYP4F3A enzyme in the strain YAB79::4F3A(HIS3,URA3), referred to as 4F3A(HIS3,URA3) in Figure 5.26, microsomal CYP4F3A enzyme was isolated. Both strains were grown in 400 ml of YPD cell culture, under exactly the same conditions. The correctly folded CYP protein, which provides the activity, was determined at 450 nm via CO difference spectroscopy (see Chapter 2, Section 2.8.3.1). Results shown in panels (A) and (B) of Figure 5.27 clearly show that the amount of enzyme produced by this integrated strain is more than the enzyme obtained from the episomal plasmid containing strain YAB79::4F3A(2µ), referred to as 4F3A(2µ) in Figure 5.27.

Panel C (Figure 5.27) shows that the strain containing 2 integrated copies of the *CYP4F3A* gene produced 56 nanomoles of CYP4F3A enzyme while the episomal produced 5 nanomoles from 400 ml of cell culture. Panel D depicts a Western blot of CYP4F3A

proteins obtained from different strains. One can see that the strains which express 1-2 integrated copies of human *CYP4F3A* gene clearly produce more protein than the strain that expresses *CYP4F3A* from an episomal plasmid.

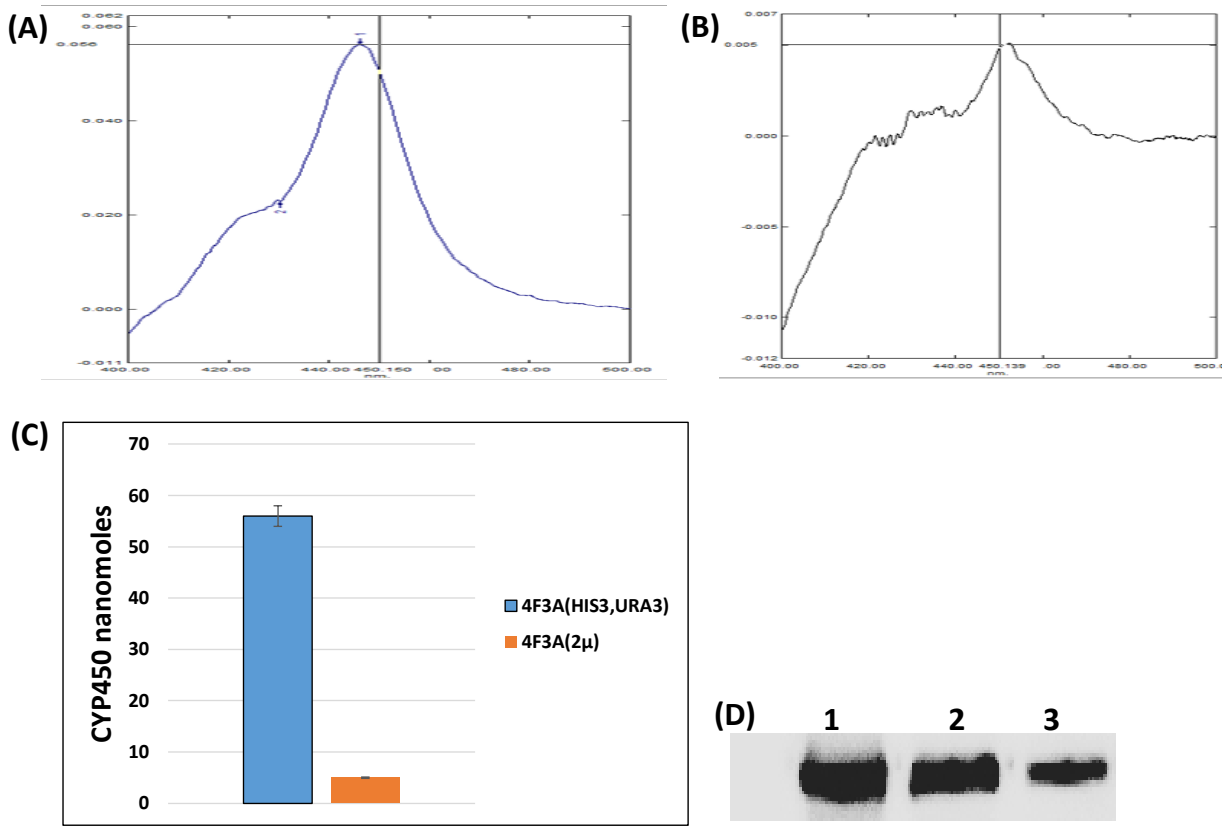


Figure 5.27. (A), (B): P450 contents, in the strains YAB79::4F3A(HIS3,URA3) [4F3A(HIS3,URA3); (A)] and YAB79::4F3A(2µ) [4F3A(2µ); (B)] were determined by CO difference spectroscopy using 1.0 µg of microsomal protein. (C) The amount of CYP450 (in nanomoles), obtained from the 2 strains (from 400 ml of YPD culture grown in baffled flasks), and represented as bar plots. The data represent mean ± S.D. of three independent experiments. (D) Western blots where 10 µg of total protein from the strains YAB79::4F3A(HIS3,URA3) (lane 1), YAB79::4F3A(HIS3) (lane 2) and YAB79::4F3A(2µ) (lane 3) were probed with a CYP4F3A specific antibody (Santa Cruz Biotechnology, Cat no:sc-374421).

5.3.21 Comparison of enzyme activity of CYP1A2 Sacchrosomes with commercially available CYP1A2 microsomes isolated from insect and bacterial cells

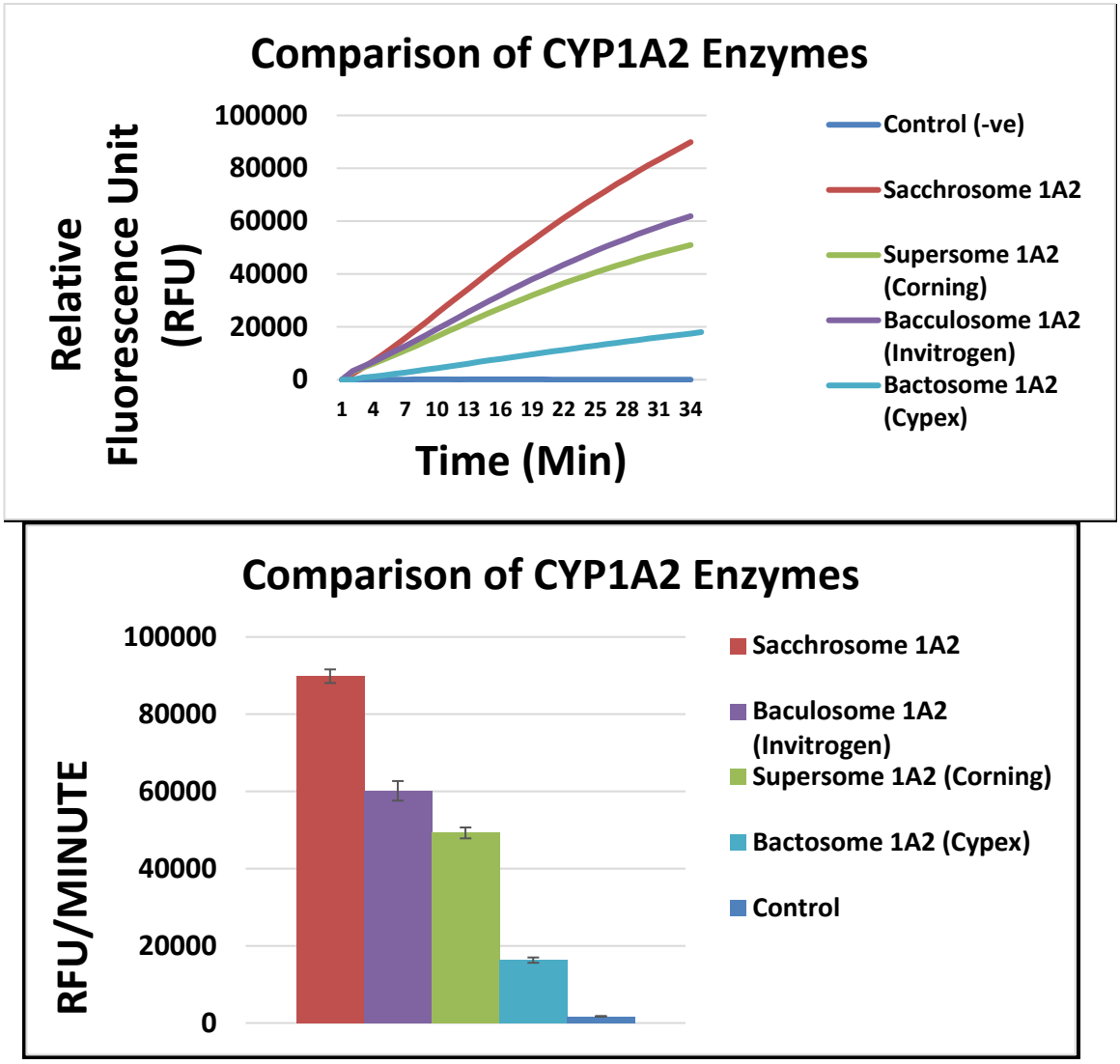


Figure 5.28. 1 picomole of CYP1A2 microsomes isolated from yeast (Sacchrosomes), insect cells [Baculosomes (Invitogen); Supersomes (Corning)], and bacterial cells [Bactosome (Cypex)]. Substrate concentration used was 4 µM CEC (3-Cyano-7-ethoxycoumarin) for each assay. The data represent mean ± S.D. of three independent experiments.

Results show that CYP1A2 Sacchrosomes are ~40% better in activity than the best commercially available enzyme, Baculosome, available from Invitrogen (Thermo-Fisher).

5.3.22 Comparison of enzyme activity of CYP2C9 Sacchrosomes with those of commercially available CYP2C9 microsomes from insect and bacterial cells

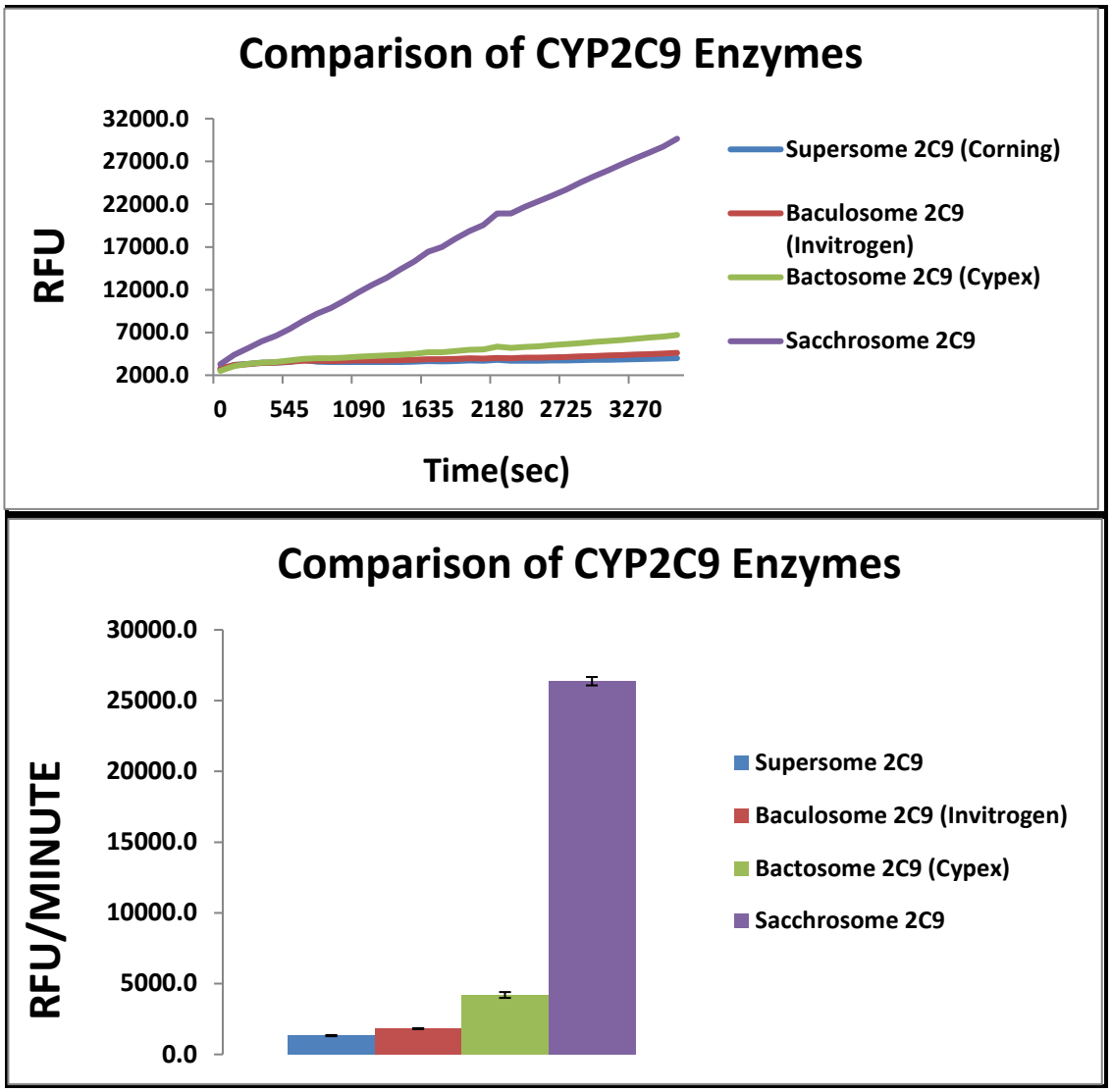


Figure 5.29. 1 picomole of CYP2C9 microsomes isolated from yeast (Sacchrosome), insect cells [Baculosome (Invitogen); Supersome (Corning)], and bacterial cells [Bactosome (Cypex)]. Substrate concentration used was 10 µM 7-MFC (7-Methoxy-4-trifluoromethylcoumarin) for each assay. The data represent mean ± S.D. of three independent experiments.

Results show that CYP2C9 Sacchrosomes are 6-fold better in activity than the best commercially available enzyme, Bactosome, available from Cypex.

5.3.23 Comparison of enzyme activity of CYP2C19 Sacchrosomes with those of commercially available CYP2C19 microsomes from insect and bacterial cells

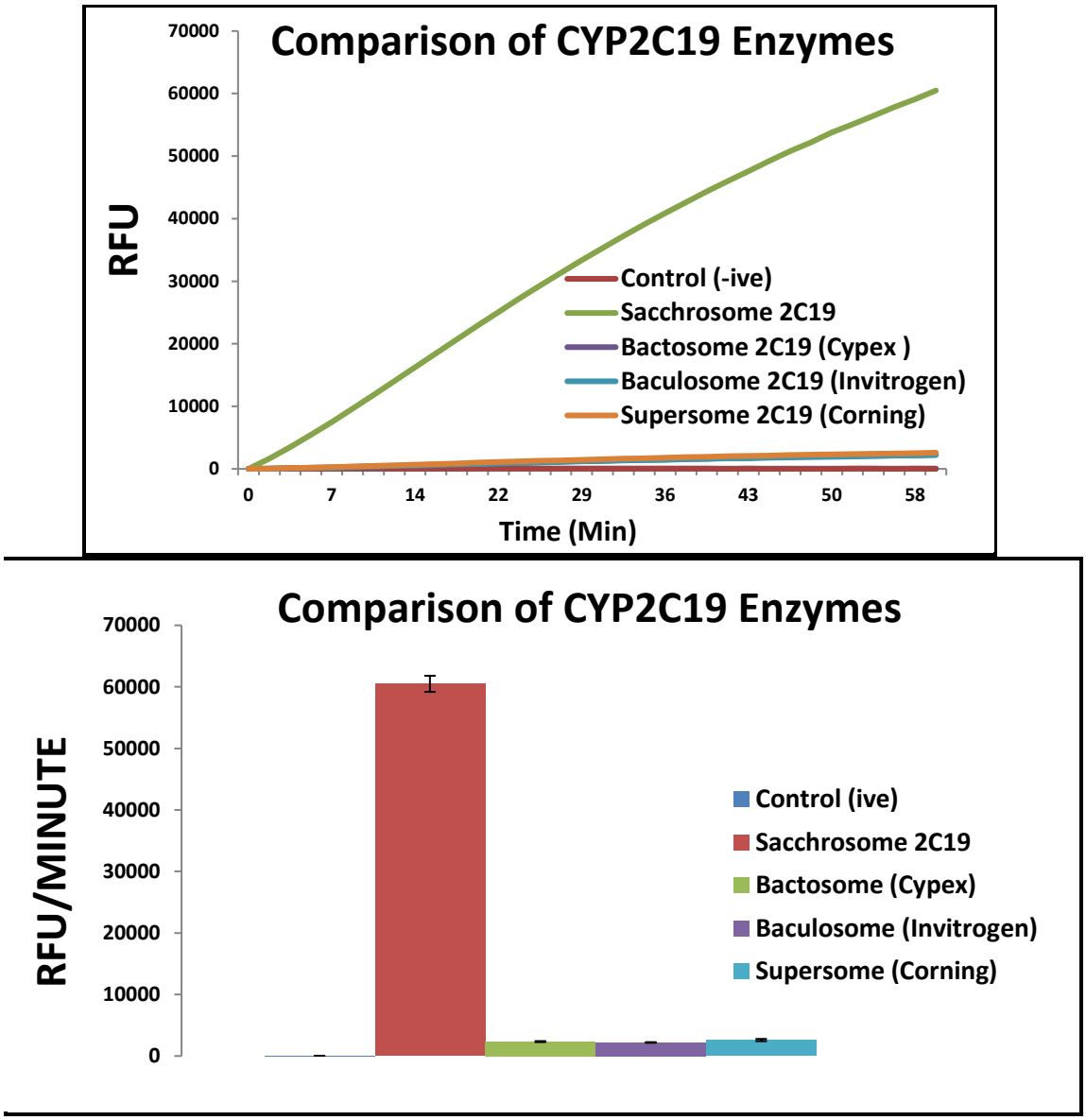


Figure 5.30. 1 picomole of CYP2C19 microsomes isolated from yeast (Sacchrosomes), insect cells [Baculosomes (Invitogen); Supersomes (Corning)], and bacterial cells [Bactosomes (Cypex)]. Substrate concentration used was 4 µM CEC (3-Cyano-7-ethoxycoumarin) for each assay. The data represent mean ± S.D. of three independent experiments.

Results show that CYP2C19 Sacchrosomes are 25-fold better in activity than the other three commercially available enzymes available from Corning, Cypex and Invitrogen.

5.3.24 Comparison of enzyme activity of CYP2D6 Sacchrosomes with those of commercially available CYP2D6 microsomes from insect and bacterial cells

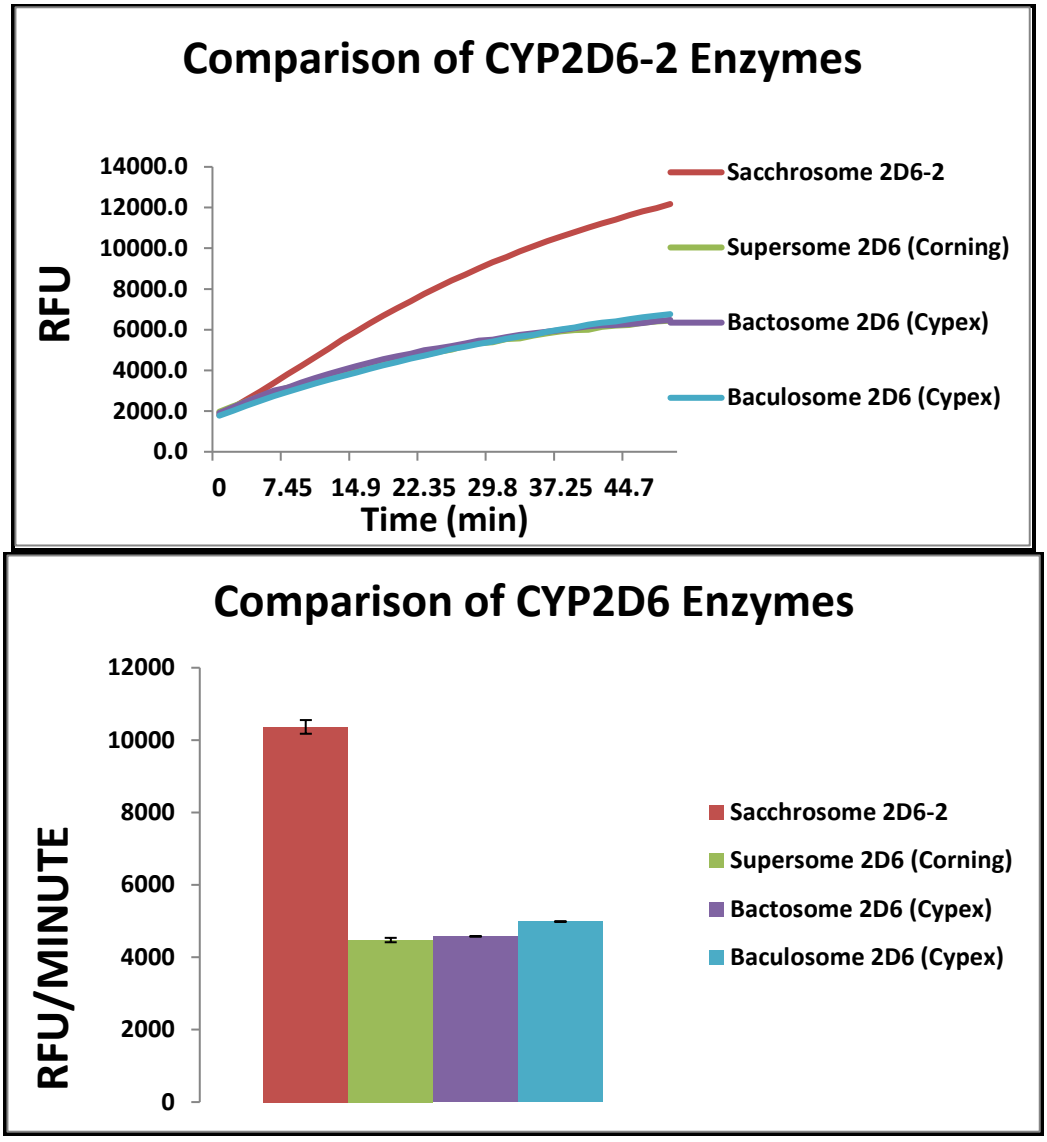


Figure 5.31. 1 picomole of CYP2D6-2 microsomes isolated from yeast (Sacchrosomes), insect cells [Baculosomes (Invitogen); Supersomes (Corning)], and bacterial cells [Bactosomes (Cypex)]. Substrate concentration used was 4 µM EOMCC (7-Ethyloxymethyloxy-3- cyanocoumarin) for each assay. The data represent mean ± S.D. of three independent experiments.

Results show that CYP2D6-2 Sacchrosomes are at least 2-fold better in activity than the other three commercially available enzymes available from Corning, Cypex and Invitrogen.

5.3.25 Comparison of enzyme activity of CYP3A4 Sacchrosomes with those of commercially available CYP3A4 microsomes from insect and bacterial cells

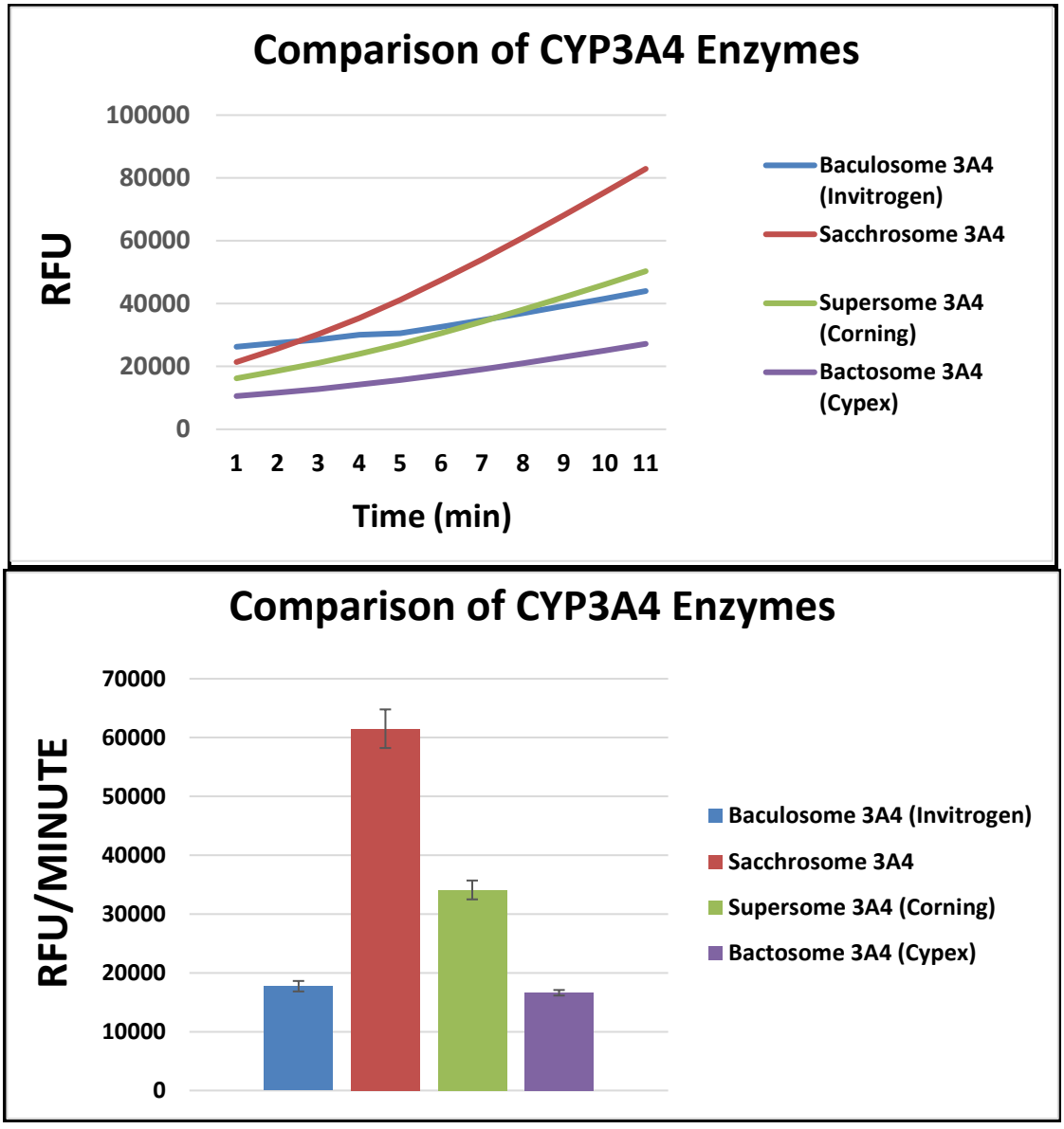


Figure 5.32. 1 picomole of CYP3A4 microsomes isolated from yeast (Sacchrosomes), insect cells [Baculosomes (Invitogen); Supersomes (Corning)], and bacterial cells [Bactosomes (Cypex)]. Substrate concentration

used was 10 µM BOMCC (3-cyano-7-(benzyloxymethoxy)-coumarin) for each assay. The data represent mean ± S.D. of three independent experiments.

Results show that CYP3A4 Sacchrosomes are ~2-fold better in activity than the enzyme (Supersome) available from Corning.

5.3.26 Comparison of enzyme activity of CYP2E1 Sacchrosomes with one of the commercially available CYP2E1 microsomes from insect cells

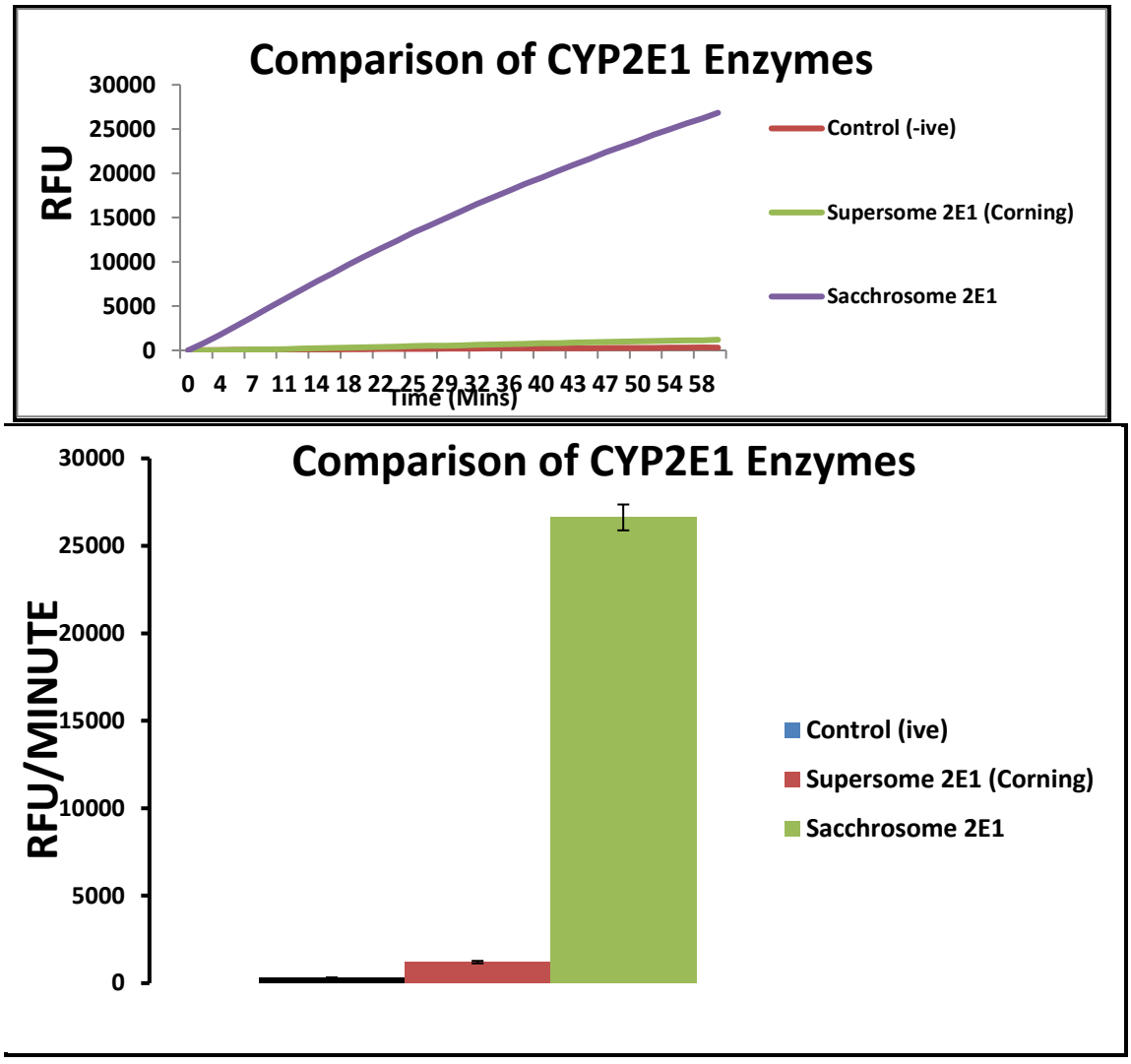
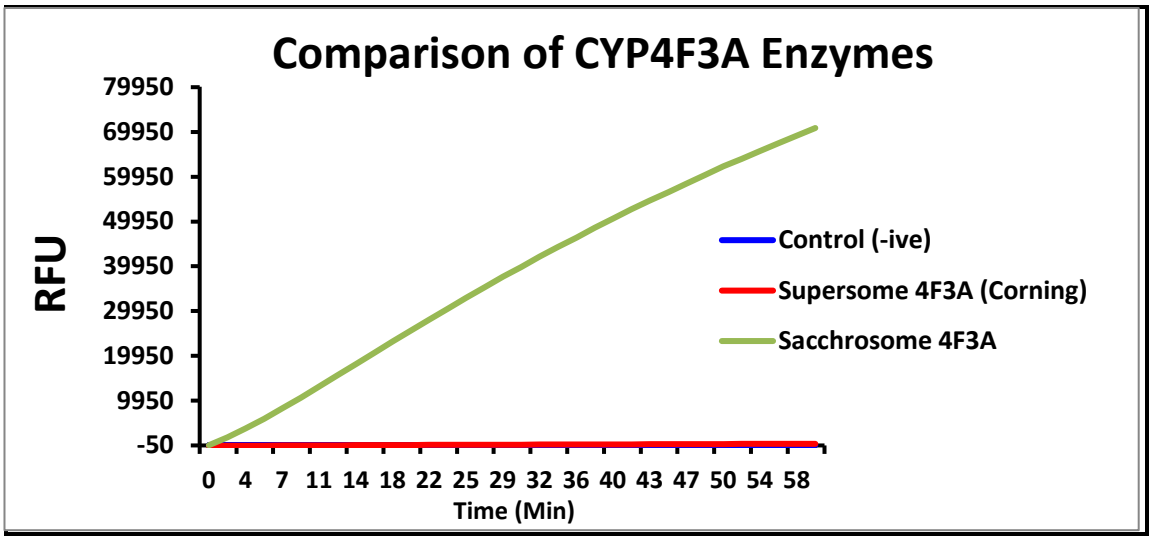


Figure 5.33. 1 picomole of CYP2E1 microsomes isolated from yeast (Sacchrosomes), insect cells [Baculosomes (Invitogen); Supersomes (Corning)], and bacterial cells [Bactosomes (Cypex)]. Substrate concentration

used was 10 µM EOMCC (7-Ethyloxymethyloxy-3- cyanocoumarin) for each assay. The data represent mean ± S.D. of three independent experiments.

Results show that CYP2E1 Sacchrosomes are at least ~20-fold better in activity than the enzyme (Supersome) available from Corning.

5.3.27 Comparison of enzyme activity of CYP4F3A Sacchrosomes with the only commercial CYP4F3A microsomes available from insect cells



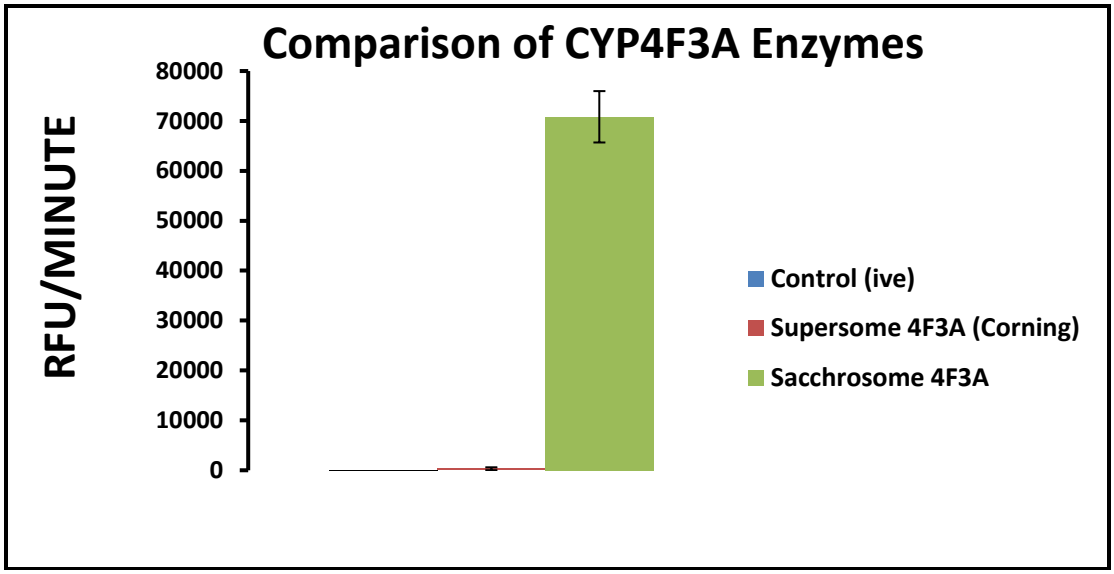


Figure 5.34. 1 picomole of CYP4F3A microsomes isolated from yeast (Sacchrosomes), insect cells [Baculosomes (Invitogen); Supersomes (Corning)], and bacterial cells [Bactosomes (Cypex)]. Substrate concentration used was 4 µM CEC (3-Cyano-7-Ethoxycoumarin) for each assay. The data represent mean ± S.D. of three independent experiments.

Results show that CYP4F3A Sacchrosomes are ~200-fold better in activity than the enzyme (Supersome) available from Corning-Gentest.

5.3.28 Epilogue: CYP microsomal enzymes

In general, recombinant DNA technology has been very helpful in the discovery of new *CYP* genes. The use of purified CYP microsomal enzymes (i.e. enzymes bound to endoplasmic reticular membranes), from recombinant systems, and the identification of selective CYP substrates with their inhibitors has been a work in progress over the past few decades.

Microsomal CYP enzymes have been used to delineate the role human CYP enzymes play in the solubilisation of prospective new medicines for excretion from the human body, a process known as drug metabolism. To be able to achieve these goals, the pharmaceutical industry constantly uses these recombinant CYP enzymes to examine drug metabolism in vitro. Before studies in animals begin, recombinant CYP enzymes, derived from insect and bacterial cells, currently provide an ethical and cost effective means to investigate drug metabolism (Rodrigues, 1999). They are used to define, in vitro, the CYP-mediated metabolites that are formed within the human body (Masimiremba at al., 1999). Gonzalez and Korzekwa (1995) have explained that it was vital to express human CYP enzymes in heterologous expression systems. In their review of the topic,

they also have claimed that the activities of recombinant human CYP enzymes were comparable to that present in human liver microsomes where multiple CYPs exist at the same time.

In contrast to pools of human liver microsomes which have huge batch to batch variations in CYP activities, recombinant CYP enzymes can be manufactured reproducibly in large amounts to (a) meet the demands of screening large chemical libraries of compounds for inhibition of CYPs, and (b) obtain metabolic products, from prospective medicines in development, for further characterization.

In this Chapter, results have been presented that show the creation of stable yeast strains that allow production of large amounts of CYP enzymes from yeast. These enzymes have been named Sacchrosomes, that is, microsomes isolated from the yeast *Saccharomyces cerevisiae*. These enzymes have been shown to have activities that surpass the activities of currently available commercial enzymes.

5.4 Discussion

In this chapter, the role of chromosomal copy number of *CYP* genes in the expression of CYP enzymes in bakers' yeast has been established. The CYP isoforms that are involved in the metabolism of most pharmaceuticals are CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Baranczewski, 2006 and Schroer, 2010). They were produced in high levels and activities in yeast, amongst many other CYP isozymes, using two copies of their genes integrated at two different chromosomal loci, *HIS3* and *URA3*.

Inhibition studies with these enzymes are essential for establishing the propensity for drug-drug interactions of prospective medicines (Schroer, 2010 and Tracy, 2016). These inhibition studies are usually performed at a late stage of the process of drug discovery because recombinant CYP enzymes are very expensive. It would be immensely advantageous if these enzymes were to be available at the early stage of drug discovery. It would allow elimination of possible drug-drug interactions early on in the drug discovery process and would significantly reduce the risks involved in the discovery of new drugs. It would also lead to diminishing the huge expenditures that pharmaceutical companies face during the process of approval of a new chemical entity (NCE) by drug regulatory authorities. Recombinant CYP enzymes which are reproducibly high in their activity and which could be cheaply available, as described in this Chapter, would be a boon to drug discovery.

Selleck et al (2008) has revealed certain bacterial cell lines for the production of CYP enzymes. It is widely accepted that bacterial cells are easy to grow and can make large amounts of recombinant proteins which may or may not be active. But contrary to this

report, as shown in this Chapter, baker's yeast has also proved to be quite exceptional in the production of CYP enzymes, especially using *CYP* genes synthesised using yeast biased-codons. Selleck et al (2008) also reported a strategy whereby expression of recombinant CYPs can be enhanced in bacterial (*E. coli*) cells through modification of the 5'-end of the *CYP* genes so that strong secondary sequences at the N-terminus, around the initiation codon of human CYP proteins, are deleted. It was also observed that for expression of active human CYPs in bacterial cells, co-expression of a CYP and a cytochrome P450 reductase (CPR) was essential. Co-expression enhanced the enzymatic activity of CYP3A4 30-fold compared to parent bacterial strain that did not contain CPR.

In the previous Chapter of this study (Chapter 4), it was shown that co-expression of cytochrome b5 may enhance enzyme activity of a certain isozyme, CYP1A2, which was not known to require cytochrome b5 for its activity. For CYP2D6, CYP1B1 and CYP1A1 enzyme activity, when expressed in baker's yeast, it was confirmed that cytochrome b5 is not required. The data for CYP1B1 and CYP1A1, in the presence of cytochrome b5, has not been shown but is similar to the data shown in Chapter 4 (Figure 4.45).

The enzymes CYP2C19, CYP2C9, CYP2C8, CYP3A4, CYP3A5, CYP2E1 and CYP4F3A studied in this report have shown higher levels of activity and protein when co-expressed with cytochrome b5 (results not shown).

Table 5.2 compares the essential features of the yeast recombinant system, presented here, and other recombinant systems that have been published.

Table 5.2. Comparison of expression of human CYP genes, chemically synthesised using yeast biased codons and integrated at chromosomal loci of bakers' yeast with that obtained from other cell systems (Punaptre at al., 2008).

Expression system	CYP Production Time	Average CYP Enzyme Yield	Strengths & Weaknesses
Bakers's yeast *(S. cerevisiae* (using *CYP* genes synthesised using yeast biased codons)	4 days	> 200 nmol/L from cells grown in 400 ml cultures grown in 2L baffled shake flasks. Yields are likely to be much higher in fermentors	Ease of growth and purification; process similar to that in mammalian cells due to the ability to localize CYP proteins on ER membranes. Unlike bacterial expression, no requirement of any modification in the CYP genetic sequence. Chromosomal integration of CYP genes creates stable cell lines. Provides highly active CYPs for in vitro studies. Cost effective for large-scale production.
Bacterial (*E. coli*) cells (using *CYP* genes with major modifications of their DNAsequences)	3 months	150 nmol/L from cells grown in large-scale, in fermentors	Loss of plasmid stability when grown for a longer period of time. CYP proteins produced not the same as wild type.
Insect cells	> 3 months	100 nmol/L from cells grown in large fermentors	Lower expression levels. Production much more expensive than yeast and bacterial cells.
Mammalian cells	6 months	100 pmol/mg from cells grown in large bags	Longer time for synthesis of CYP proteins; low expression levels and expensive.

In general, all CYP enzymes studied have been expressed from *CYP* genes that have been chemically synthesised using yeast biased codons. Optimal reproducible yields were

obtained only when the CYP gene expression cassettes are permanently integrated into specific locations on yeast's chromosomes.

Several recent studies have shown the importance of codon bias such as the existence of a 'ramp' of rare, slowly translated codons at the 5' end of a protein-coding sequence, which help in the efficiency of of protein synthesis (Tuller et al., 2015). Pechmann and Frydman (2013) established that codon bias influences protein folding during expression. In another study, Ingolia (2014) has revealed that codon bias plays a vital role in translation of ribosome profiling data and this helps in translation efficiency.

Human liver microsomes (HLMs) are preferred in pharmaceutical industry for CYP inhibition studies. HLMs are derived mostly from human livers obtained from individuals who are alcoholics and drug addicts. These degenerate livers are the major source of HLMs and hepatocytes which are used to obtain vast amount of data that is later evaluated by the FDA for their final decision regarding approval of an NCE. It would definitely be worthwhile if alternative systems were available which could be used instead of HLMs.

The invention of CYP microsomal enzymes, using cDNAs optimized for expression in bakers' yeast can greatly facilitate CYP inhibition studies ethically and in a much more cost effective manner. Three companies that manufacture CYP microsomal enzymes are Cypex (Bactosomes, isolated from bacteria), Invitrogen, a Thermo-Fisher subsidiary (Baculosomes, from insect cells) and Corning (Supersomes from insect cells). The IC_{50} values of inhibition of microsomal enzymes (Sacchrosomes and Supersomes/ Baculosomes), for the five major CYPs by FDA-approved CYP inhibitory compounds, have been compared with inhibition of CYP enzymes residing in HLMs. The comparative data is depicted in Table 5.3.

Table 5.3. Comparison of IC_{50} values of CYP inhibitory compounds for inhibition of CYP enzymes expressed in yeast with that published in the literature.

CYP	Inhibitor	Fluorogenic Substrate	IC_{50} values for inhibition of CYP microsomes isolated from yeast (Sacchrosomes)	IC_{50} values for inhibition of CYP microsomes isolated from insect cells (Supersomes/ Baculosomes)	Published IC_{50} values for inhibition of CYPs in HLMs
1A2	Furafylline	CEC	3.6 µM ± 0.001	0.5 µM ± 0.2[1,2]	5.26 µM [a]
2D6	Quinidine	EOMCC	0.69 µM ± 0.002	0.01 µM ± 0.02[1,2]	0.47 µM [b]
2C19	Ticlopidine	CEC	0.26 µM ± 0001	0.01 µM ± 0.02[1,2]	0.23 µM [c]
3A4	Ketoconazole	DBF	0.24 µM ± 0.03	0.02 µM ± 0.02[1,2]	0.18 µM [d]
2C9	Sulphaphenazole	DBF	0.9 µM ± 0.002	0.24 µM ± 0.15[1,2]	0.5 µM [d]

IC_{50} values published in Corning and Invitrogen websites.
[a]Lin et al, 2007; [b]Ghosal et al, 2003; [d]Guidance for Industry, 2012; [c]Stresser et al, 2004 (IC_{50} values in HLMs).

These CYP enzyme inhibition studies (Table 5.3) were performed to gauge the validity of the yeast-produced enzymes. The closer the IC_{50} values are, using isolated microsomal CYP enzymes, to the values obtained using HLMs would imply that the recombinant enzymes have similar 3-D structures at their active sites to the native enzyme. The IC_{50} value for CYP inhibition by a compound denotes the concentration of compound that allows 50% inhibition of activity of an enzyme. Different IC_{50} values, using different

enzymes form different recombinant sources, would signify that active sites structures of the enzymes are likely to be dissimilar to the native enzyme. Failing to portray the true CYP inhibitory capacity of a compound in pre-clinical studies can lead to major safety concerns in clinical development and have, in the past, led to the removal of several drugs from the market (Diaz, 2016).

The results in Table 5.3 reveal that the active sites of the CYP-Sacchrosomes are probably quite similar to the native enzymes in HLMs. It would indicate that most Sacchrosomes possess active sites that are probably identical to the native enzymes produced in human liver cells. Hence, we would like to propose that Sacchrosomes could reliably be used, instead of HLMs, for rapid screening of CYP inhibitory compounds.

Chapter 6 Biotransformation using recombinant CYP-expressing baker's yeast cells

6.1 Background

6.1.1 Whole yeast cells for biotransformation

The endogenous enzymes existing within baker's yeast has been described as being ideal for chemists in their search for stereo-selective biocatalysts which could lead to the syntheses of novel and important chemical compounds (Servi, 1990). Yeast is an organism which is non-pathogenic, inexpensive and simple to culture in the laboratory. Moreover, its growth can be optimised for large-scale production of cells. Stocks of yeast strains can be stored indefinitely at 4°C as agar stabs or at -80°C as glycerol stocks (Faber *et al*., 2004).

There are three major advantages of using whole native baker's yeast cells for biocatalysis.

(1) The cellular abundance of all required cofactors of important enzymes that could be envisaged to participate in biotransformation and the presence of metabolic pathways for their regeneration.

(2) The ability of yeast cells to grow vigorously in glucose, which is inexpensive and which is used by yeast as an energy source and as well as a substrate of enzymes essential for growth.

(3) The compounds which are recovered from yeast cell culture after biocatalysis can be used without the necessity of protracted purification from toxic metabolites which occur in organisms other than yeast (Mata-Gomez et al., 2014).

6.1.2 Recombinant CYP-expressing yeast cells for possible biotransformation reactions

Recombinant CYP450-expressing baker's yeast cells have the potential of being used for redox biocatalysis, provided the cells efficiently express heterologous CYP enzymes. Efficiency of expression relates to how much of CYP enzymes are made within the cells and how active they are. In theory, yeast cells should provide a comfortable environment for the proteins that are expressed within the cells making them more stable. The caveat, however, is that the overproduction of heterologous proteins usually induces proteolysis of the proteins that are being expressed (Prelich, 2012).

In order to avoid degradation of foreign proteins expressed in yeast, basic yeast strains can be selected for resistance to proteolysis of specific proteins. In fact, that's how BC300, derived from the commercially available ATCC strain W303-1A (ATCC #208352), was selected for production of heterologous membrane proteins over many years. BC300 is the strain that has been used for all the work described in this thesis. BC300 was identified many years before the work described in this thesis began.

This Chapter focusses on the use of recombinant CYP-expressing whole yeast cells for biotransformation. In nature, important metabolites are often produced via inefficient natural biological processes, resulting in their scarcity. The complexities of the structures

of naturally occurring molecules make it very difficult for them to be synthesised easily. Around a couple of decades ago, it was first thought that natural yeast cells themselves could be used for biotransformation processes. It was surmised that endogenous yeast enzymes, similar to those involved in the synthetic pathways of certain metabolites, would be helpful in converting a natural product A to another natural product B, “B” not being amenable to easy isolation (Kebamo et al, 2015).

6.1.3 Syntheses of secondary metabolites via bioorganic reactions

Some secondary metabolites, such as morphine, taxol, artemisinin, coenzyme Q10, docosahexaenoic acid (DHA), and carotenoids, which exist in plants, mammals, microalgae, and other microorganisms (Facchini et al., 2012) are regarded as high-value metabolites (Chang and Keasling, 2006). Vast majority of these metabolites play important roles in coping with or reacting to environmental changes by the human organism. The metabolites provide protection against biotic and abiotic stresses, radiation, and may at times act as regulatory molecules for human health (Marienhagen and Bott, 2013). All these metabolites are, in one way or the other, involved in human health and treatment of diseases. For example, morphine is used for pain control (Chappell, 2008), taxol and vincristine for cancer treatment (Chappell, 2008 and Ye and Bhatia, 2012), and carotenoids and DHA as part of heart disease treatment (Marta-Gomez et al., 2014).

Up to 30-40% of the FDA approved drugs have been derived from natural metabolites. More than 48% of drugs used in cancer research stem from naturally occurring

metabolites (Li and Vederas, 2009; Newman and Cragg, 2012). These metabolites are present in very low quantity in natural sources. Ye and Bhatia (2012) state that less than 0.2% of the total dry weight yield of the Pacific yew trees consists of taxol. The content of vincristine in *Cantharanthus roseus* is only 0.0003% (Kuboyama et al., 2004). It is obvious that these low quantities in the natural sources place barriers to the industrial use of these very important metabolites (Chang and Keasling, 2006).

The approaches to organic chemical synthesis of high-value, scarcely-occurring natural products have been hindered by low yields in multi-step synthetic protocols which can be also be tedious or impossible to achieve for production of large amounts of a particular chemical (Nicolaou et al., 1994; Kuboyama et al., 2004). For years, baker's yeast has been used in the production of different types of food and beverages. It is also well recognised that baker's yeast is safe for human consumption.

6.1.4 Using yeast to perform CYP450-mediated bioorganic reactions

Since the genome of baker's yeast *Saccharomyces cerevisiae* has been sequenced completely (Goffeau, 2000), the revelation of the genome's DNA sequence can now play a key role in research related to yeast molecular biology. For production of high-value metabolites, baker's yeast has genetically been engineered so that it could be used as a cell factory for exploration of new methods that would allow synthesis of important chemicals of value (Shao and Zhao, 2009). Creation of these new methodologies involved expression of heterologous proteins in yeast (Dai et al., 2012; Zhou et al., 2012).

During the study conducted for this thesis, it was observed that biosynthesis of human CYP proteins in yeast can be stabilized when expressed from genes that had been chemically synthesised using yeast biased codons. Upregulation of levels of active CYP enzymes was seen compared to enzymes that were produced from native genes that had been isolated from a human liver cDNA library (Chapters 3 to 5). Due to yeast cells' robustness and similar intracellular architecture to human cells, baker's yeast as a recombinant organism provides a physical and physiological environment for the functional expression of any protein that is present in humans or animals. Specifically for the human CYPs which are naturally bound to the endoplasmic reticular (ER) membranes, yeast provides the same ER membranes which have similar structure to the ER membranes present in human cells. Therefore, human CYP proteins can be anchored to the ER membranes of yeast, to possibly provide similar activity to that present in human cells (Eckart and Bussineau, 1996; Pompon et al., 1996).

In recent years, the discovery of genes, coding for membrane-bound proteins, involved in nature in the synthesis of high-value metabolites together with advances in synthetic biology has enabled successful construction of yeast strains that allow for the production of diverse sets of metabolites.

6.1.5 Yeast as a cell factory for the syntheses of steroids, – involvement of CYP450-mediated bioorganic reactions

Steroids are molecules bound by four carbon rings. They are mostly synthesised in plants, animals and fungi via CYP450-mediated chemical reactions (de Souza at al., 2011). Steroids vary from dietary lipids to cholesterol to sex hormones such as estradiol and testosterone, and the pharmaceutical drug hydrocortisone (Heftmann, 1974). Xu and Nes (1998) revealed that an erg6-mutant yeast strain besides predominantly producing ergosterol as a major metabolite also produces trace amounts of cholesterol. Presence of either of the two yeast genes *ERG5* or *ERG6* is essential for ergosterol synthesis. *ERG5* codes for a yeast CYP450, whereas *ERG6* is a sterol 24-C-methyltransferase. However, for medium level cholesterol production, both the *ERG5* and *ERG6* genes, which are responsible for introducing changes in the side-chain of sterols, had to be disrupted. Overproduction of cholesterol in yeast was only achieved by engineering the overexpression of heterologous *Danio rerio* genes that code for the enzymes dehydrocholesterol-7-reductase (DHCR7) and dehdrocholesterol-24-reductase (DHCR24) in yeast strains which lacked *ERG5* and *ERG6* genes (de Souza et al., 2011).

Hydrocortisone is an anti-inflammatory drug which is widely used as an ointment and is prescribed for oral and intravenous administration (Menkin, 1954). Yeast was used as a cell factory to synthesise hydrocortisone after the introduction of 13 genes, most of them coding for *CYP* genes that exist in plants and humans into the baker's yeast, *S. cerevisiae*. The newly introduced 13 genes were critical for the efficient synthesis of hydrocortisone in yeast (de Souza et al., 2011).

6.1.6 Flavonoids as substrates for CYP450-mediated bioorganic reactions

The flavonoid class of compounds are natural products formed from phenyl-propanoid structural motifs. Flavonids are considered as nutritional compounds (Siddiqui et al., 2012; Zhou et al., 2014). Yeast naturally does not produce flavonoids but it does produce the amino acids, tyrosine and phenylalanine, which are flavonoid precursors. By devising suitable systems for expression of heterologous proteins, yeast can be manipulated to express certain flavonoids. For example, the flavonoid naringenin has been produced from yeast by assembling, in yeast, four plant *CYP450* genes which convert p-coumaric acid to naringenin. The four *CYP450* genes cinnamate-hydroxylase (C4H) (from *Arabidopsis thaliana*), 4-coumarate-CoA-ligase (4CL) (from *Petroselinum cripum*), chalcone isomerase (CHI) and chalcone synthase (CHS) (from a *Petunia* hybrid) were introduced into baker's yeast *S. cerevisiae* resulting in a yeast strain that produced the naturally occurring flavonoid, naringenin (Yan et al., 2005).

Naringenin can be a valuable precursor for the CYP450-mediated synthesis of a variety of other flavonoids/isoflavonoids such as genistein (an isoflavone), apigenin (a flavone), kaempferol and quercetin (both flavonols) (Siddiqui et al., 2012; Zhou et al., 2014).

6.1.7 Alkaloids as substrates for CYP450-mediated bioorganic reactions

Plant alkaloids belong to a class of natural products which is highly diverse in their chemical structure. They are classified based on their heterocyclic ring system. It is estimated that, in plants, there are 12,000 different alkaloids produced with an assorted range of pharmacological properties. The alkaloids are synthesised within plants with the help of plant CYP450 enzymes. Usually, in each plant species there are 400-500 *CYP* genes whereas in humans there are only 57 *CYP*s.

6.1.8 Role of human CYP450 enzymes in drug metabolism

The human cytochrome P450 (CYP) enzymes play important roles in the metabolism of many endobiotics (i.e. molecules endogenous to the human system) and xenobiotics (i.e. molecules exogenous to the human system). Human CYP enzymes are also used for testing new drug candidates for their ability to interact with these enzymes during the preclinical development phase. Both inhibition and induction of CYP enzymes may have deleterious effects on the human body.

The human CYP isoforms that metabolise the majority of xenobiotics, that include medicines, belong to CYP1, CYP2, CYP3 sub-family of isoforms. These enzyme sub-families are broadly related to each other and have overlapping substrate specificities. Because of this, they are responsible for the metabolism of the majority (i.e. 70-80%) of known (i.e. approved) drugs (Nobert and Russell, 2002; Guengerich et al., 2005).

The pharmacokinetic behaviour of a drug is determined by the metabolic product that is formed from the drug and the duration of its metabolism (i.e. reaction rate of product formation). Variations in CYP activity, manifested after enzyme inhibition or induction by pharmaceutical drugs and other xenobiotics, is often the source of drug-drug interactions. Drug-drug interactions can have adverse effects on patients and can potentially lead to the removal of a drug from the market. The usual mechanism of drug-drug interaction is through inhibition of CYP enzymes (Kalgutkar et al., 2007; Pelkonen et al., 2008). However, if a drug specifically inhibits one CYP but does not inhibit any other CYP, it can have a 'clear' pharmacodynamic profile and may have the possibility to be part of co-therapeutics, especially in the area of antiviral drug therapy.

6.2 Outline of Chapter

This Chapter explores the use of recombinant CYP-expressing whole yeast cells for biotransformation reactions. The same yeast cells, which were described earlier in Chapter 5 for isolation of microsomal enzymes, have been used. The following recombinant yeast cells have been used for biotransformation:

(a) Cells that express human CYP1A1 or CYPA2 have been used to transform

(i) Natural product chrysin to another natural product baicalein, and

(j) Natural product chrysosplenetin to an yet undefined product (results not shown);

(b) Cells that express variants of human CYP2D6 have been used to convert the opiate codeine to the more potent opiate, morphine; and

(c) Cells that express human CYP3A4 have been used to study the metabolism of compound AZD-2014, an Astra-Zeneca anticancer drug [an mTORC (mammalian target of rapamycin complex) inhibitor] currently in multicentre Phase II/Phase III clinical trials.

It has been reported that, until now, whole cell mediated biotransformation reactions generally yield, at best, no more than 10-15% of the product (Das-Bradoo et al., 2004; Duran et al., 2017). The natural cells (i.e. not recombinant) that were used for biotransformation were from a variety of different organisms (Duran et al., 2017). Human/ non-human CYP-expressing bacterial cells have also been used with similar yields of products.

Our results, using human CYP-expressing yeast cells clearly show that there is greater than 80% product formation. The amounts of product formed were estimated from thin layer chromatographs (TLCs) and, in one case, liquid chromatograph/ mass spectrometric analysis (LC/MS).

The high yields of bioorganic reactions, using CYP-expressing yeast cells indicate that, in future, organic chemical reactions in pre-clinical drug discovery research could also be performed using this type of robust whole yeast cells. In a chemical laboratory, cells harbouring human or plant CYPs could routinely be used for small and large-scale chemical reactions that require regio- and stereo-selective organic syntheses.

6.3 Biotransformation of chrysin to baicalein: selective C6-hydroxylation of 5,7-dihydroxyflavone using whole yeast cells stably expressing human CYP1A1 enzyme

[Ibidapo S Williams et al; Journal of Agricultural & Food Chemistry (an American Chemical Society Journal), published online, August 2017; in print, in 8 weeks' time].

6.3.1 Abstract

Naturally occurring polyphenolic compounds are of medicinal importance because of their unique antioxidant, anticancer and chemopreventive properties. Baicalein, a naturally occurring polyhydroxy flavonoid possessing a diverse range of pharmacological activities, has been used in traditional medicines for treatment of various ailments(J.Mu et al., 2016). Apart from its isolation from natural sources, its synthesis has been reported via multi-step chemical approaches. Here we report a preparative-scale biotransformation, using whole yeast cells stably expressing human cytochrome P450 1A1 (CYP1A1) enzyme, that allows regio-selective C6-hydroxylation of 5,7-dihydroxyflavone (chrysin) to form 5,6,7-trihydroxyflavone (baicalein), (Zhang et al., 2014). Molecular modelling reveals why chrysin undergoes such specific hydroxylation mediated by CYP1A1. More than 92% reaction completion was obtained using a shake-flask based process that mimics fed-batch fermentation. Such highly efficient selective hydroxylation, using recombinant yeast cells, has not been reported earlier. Similar CYP-expressing yeast cell-based systems are likely to have wider applications in the syntheses of medicinally important polyphenolic compounds.

6.3.2 Introduction

Baicalein (5,6,7-trihydroxyflavone) was originally isolated from the roots of *Scutellaria baicalensis* (Chinese skullcap) which is used for treatment of chronic hepatitis, inflammatory diseases, tumours, and diarrheal in China, Korea, Taiwan, and Japan (Papafragkakis *et al*., 2016). Its isolation has also been reported from another plant, *Oroxylum indicum* (Indian trumpet flower). (Roy at al., 2007) Baicalein is known for its anticancer properties in various cancer types (Roy at al., 2007; Ma et al., 2005) including pancreatic, gastric and colorectal cancers, multiple myeloma, head and neck cancer, and breast cancer(Liu et al ., 2016; Gao et al., 2016). Baicalein is also considered an anti-inflammatory *(Li et al., 2016; Patwardham et al., 2016)*that generally protects against oxidative stress *(Tsai et al., 2016),* more specifically in cardiac cells, *(Zhao et al., 2016)* and in cisplatin-induced acute kidney injury. Because of its wide-ranging medicinal applications, an industrial scale protocol for its production would be considered as important *(Sahu, 2015).*

Baicalein has primarily been produced via five approaches: (a) single-step hydrolysis of the natural product baicalin, a glycoside, (Zhang et al., 2014) (b) 4-step total synthesis from a cinnamic acid derivative(Kim et al., 2012), (c) 4-step total synthesis from 3,4,5-trimethoxyphenol, (Chen et al., 2010), (d) 3-step total synthesis from 2,4,5,6-tetrahydroxyacetophenone, ,(Vyas et al., 2016) and (e) 6-step synthesis from chrysin, (Righi et al., 2010) involving methylation, bromination, acylation, nucleophilic replacement of Br with OMe, followed by de-acylation and demethylation as key steps.

Selective transformation, using efficient biocatalysts, of a low value phytochemical to high value phytochemical is an exciting area of bioorganic chemistry. Sordon and

coworkers have reported biotransformations of natural flavonoids naringenin, hesperetin, chrysin, apigenin, luteolin, quercetin, epicatechin, and biochanin A using the natural yeast, *Rhodotorula glutinis*.

One such approach produced norwogonin (5,7,8-trihydroxyflavone) from chrysin (5,7-dihydroxyflavone). Here we report a preparative scale process for biotransformation of chrysin to baicalein, via selective C6-hydroxylation using recombinant human cytochrome P450-1A1 (CYP1A1) enzyme expressed within baker's yeast (*Saccharomyces cerevisiae*) cells. This is the first single-step protocol for a high-yield conversion of chrysin to baicalein and, therefore, may serve as a simple and cheap strategy for production of baicalein in an industrial scale.

6.3.3 Experimental Section

General Experimental Procedures. All chemicals were obtained from Sigma-Aldrich and were used as received. H NMR spectra were recorded on Brucker-Avance DPX FT-NMR 400 MHz instrument. Chemical data for protons are reported in parts per million (ppm) downfield from tetramethylsilane and are referenced to the residual proton in the NMR solvent (CD_3OD, 3.31 ppm). ESI-MS were recorded on Waters QTOF mass spectrometer.

HPLC analysis was performed on Shimadzu LC-6AD system connected with C18 column (4.6 x 25 mm, 5 µ). Mobile phase consisted of A: 0.1% formic acid and B: methanol using isocratic elution (30: 70 – A: B). Flow rate was 1 mL/min. Detection wavelength was 270 nm.

LC-MS analysis was performed on Waters Acquity UPLC. The column used was C18, 1.7 µ with dimensions of 100 x 2.1 mm (column temp. 30 °C). Binary gradient system was used. Mobile phase A consisted of 5% acetonitrile in water with 0.1% formic acid. Mobile phase B consisted of acetonitrile with 0.1% formic acid. Gradient details are: Time in min (% B concentration): 0.01 (10), 0.25 (10), 9.00 (100), 10.00 (100), 11.00 (10), 12.00 (10). PDA range: 220 nm to 400 nm; flow rate: 0.3 mL/min.

Biotransformation experiment. Yeast strains, each of which contain two copies of human *CYP1A1* or *CYP1A2* genes, downstream of the *ADH2* promoter, integrated into chromosomal loci of the genome of the yeast strain W303-1a (ATCC 208352), were used for biotransformation (Section S2 of supporting information). That they expressed CYP1A1 and CYP1A2 proteins were confirmed by Western blotting (supporting information-S4). The strains, from frozen stocks, were revived in 250 mL Erlenmeyer baffled flasks containing 50 mL YPD (Yeast, Peptone, Dextrose) medium with composition (gL^{-1}): peptone 20; yeast extract 10; glucose 15.0, pH 6.0. The flasks were shaken at 200 rpm, at 28 °C. Three consecutive YPD pre-cultures were grown for high biomass production, before addition of the substrate to cells grown in SD (Synthetic Defined) medium. Typically, loopful of CYP-containing freshly grown yeast cells was inoculated in a 500 ml Erlenmeyer baffled flask separately containing 100 ml YPD medium (pre-culture -1) at 28 °C for 24 h. The cells were harvested after 24 h and inoculated into a new 500 ml baffled flask containing 100 ml YPD medium (pre-culture -2) at 30 °C for 18 h. The process was repeated three times for the cells to reach an OD_{600}, of ~90.

The harvested cells, ~3.0 ml (OD_{600}, ~90), were inoculated in 200 ml of minimal SD medium contained in a 1 L baffled flask. Composition of SD medium (gL^{-1}): dextrose 1.0; dipotassium phosphate 7.0; monopotassium phosphate 2.0; sodium citrate 0.50; magnesium sulphate 0.10; ammonium sulphate 1.0, pH 7.0 ± 0.2 at 28 °C. Initially, reaction was carried out with 10 mg of chrysin. Later, the chrysin at different concentrations viz. 0.2, 1, 2, 5 and 10 mg/mL were dissolved in DMSO and further incubated in 50 mL of SD medium (keeping the DMSO concentration > 0.5%) for 72 h at 28°C, 200 rpm. After every 24 h, the medium was replenished with 1.5% w/v of glucose. For optimization of incubation time, the SD cell culture media were harvested after 24, 48, 72, 96, 120, 144 and 160 h, and were then analysed on TLC and HPLC/ LC-MS to monitor the yields of biotransformation at each time point.

The reaction media was extracted with ethyl acetate (3 times). The combined ethyl acetate layer was concentrated on vacuo-rotavapor to obtain crude extracts that contained the biotransformation product. The crude residue was loaded on a reverse phase (C18) silica gel column packed in water. The crude extract was loaded on the column by making a slurry with C18 silica gel. The column was then eluted with increasing concentrations of methanol in water. The desired product was collected at 50% methanol in water. Evaporation of the solvent gave a yellow solid which was characterized as baicalein (**1**). Yellow powder; m.p. 262-265 °C (Lit. 264-265 °C); TLC: Rf = 0.5 (3% Methanol in DCM) and 0.8 (40% EtOAc: Hexane with 0.1% acetic acid); ^{1}H NMR (CD_3OD, 400 MHz, δ ppm): 7.91 (dd, J = 4, 8 Hz, 2H), 7.48 (m, 3H), 6.66 (s, 1H), 6.55 (s, 1H); ESI-MS: *m/z* 271.10 $[M+H]^+$. The spectral data was identical to that reported in the literature[32] and TLC matched with reference sample obtained from Sigma-Aldrich (CAS Number 491-67-8).

Molecular modeling: The docking of chrysin with CYP1A1 (PDB ID: 4I8V) was performed using the protocol as described in our earlier publications.[36, 37]

6.3.4 Results and Discussion

CYP enzymes are known for their exceptional ability to carry out hydroxylation, epoxidation or demethylation reactions in a regio-selective fashion both in plants and humans. We have developed an efficient technology for stable expression of human CYP enzymes within yeast cells. Using this platform, recombinant baker's yeast cells can continue to express CYPs in shake flasks, over a week or more, with increasing activities, under conditions that mimic fed-batch growth (Supporting Information; S1). Microsomal CYP enzymes, isolated from these same recombinant yeast cells, have successfully been used by us earlier as drug discovery tools for screening synthetic compounds and natural product repositories to identify possible cancer chemopreventive agents.[37-39]

Selection of CYPs for biotransformation of chrysin. In this work, use of recombinant CYP-expressing whole yeast cells has been explored for their ability to be used as biocatalysts for biotransformation reactions. For one of our first experiments, we chose the flavonoid chrysin as a substrate using two yeast-expressed enzymes of the CYP1 sub-family (i.e. CYP1A1 and CYP1A2) as biocatalysts.

Two recombinant yeast strains, each containing two chromosomally integrated copies of *CYP1A1* and *CYP1A2* genes under the control of the ethanol-inducible alcohol dehydrogenase 2 (*ADH2*) promoter, were created to enable stable and reproducible biotransformation reactions (plasmid maps shown in Supporting Information, S2). The *ADH2* promoter is repressed in the presence of glucose. The aim was that recombinant

yeast cells, containing stably integrated *CYP* expression cassettes, would be grown initially in complete YPD medium (containing 2% glucose) to obtain large optical density, measured at a wavelength of 600 nm (OD_{600}) of ~90, in shake flasks. This would occur without any plasmid loss and would be achieved over 3 days through repetitive replenishment of glucose, every 24 h, in the non-selective highly nutritious YPD medium. Cells grown in YPD [Figure 6.1 (a)] would then be re-suspended in minimal selective SD medium (pH 7.0), supplemented with 1.5% of glucose, for the biotransformation reaction. It was thought that rich full YPD medium may not be appropriate for biotransformation since there is a possibility of substrate binding to its ingredients.

For initial optimization, reactions were performed in SD with 10 mg of chrysin in baffled flasks shaking at 200 rpm for 72 h, at 28 °C [Figure 6.1 (b)]. After every 24 h, the medium was replenished with fresh glucose to a final concentration of 1.5%. Glucose is exhausted after 12 h of growth of yeast cells when it is converted to ethanol. Hence, the ethanol-inducible *ADH2* promoter is fully induced every 12 h before more glucose is added to the medium for further rounds of expression.

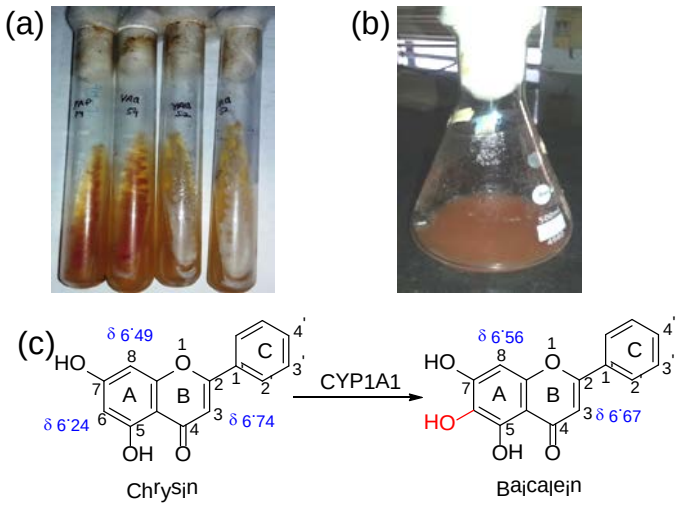


Figure 6.1. (a) CYP1A1-expressing cells grown in YPD Medium; (b) Biotransformation reaction using CYP1A1 in SD medium; (c) Structures of chrysin and baicalein showing key chemical shift values

The reaction media at each time point was extracted with ethyl acetate. The extracts were analyzed by TLC followed by HPLC/LC-MS. The formation of new product on TLC with lower Rf value than the substrate gave us an indication of a hydroxylation reaction (Supporting Information, S3). LC-MS analysis of the reaction mixture confirmed the product as mono-hydroxy chrysin with mass of *m/z* 270. The same product formed using both the enzymes, CYP1A1 and CYP1A2, expressed within yeast cells. However, conversion of substrate to product was better with CYP1A1. Therefore, for subsequent scale-up experiments, only CYP1A1-expressing yeast cells were used for biotransformation.

Scale up and optimization of biotransformation reaction. Scale-up was performed as above; cells were at first cultivated in non-selective YPD media for 72 h, with fresh glucose (2%) being added every 24 h. Cells were re-suspended in selective SD minimal medium. 10, 50, 100, 250 and 500 mg of chrysin were used as substrate for separate biotransformation reactions. The chrysin-containing cell culture media were replenished with glucose every 24 h to a final concentration of 1.5%. The reaction using 100 mg of chrysin was observed to be the most efficient. With 250 and 500 mg, incomplete biotransformation occurred, probably because the small number of cells, used for growth in these specific experiments, was inhibited by the substrate.

Based on these results, it was decided to optimize the time period for biotransformation. For this, we chose 100 mg of substrate with reaction time points of 24, 48, 72, 96, 120, 144 and 160 h. HPLC analyses of the reaction mixture after 24, 48, 72, 96, 120, 144 and

160 h incubations indicated that, at 144 h, there was nearly complete conversion (>92%) of chrysin to the product. The HPLC chromatograms of reaction mixtures at representative four time intervals are shown in Figure 6.2. In HPLC analysis, the concentration of sample injected at each time interval was kept constant. The increase in the AUC of baicalein with increase in the time, from 24 h to 144 h, is indicative of the fact that the highest amount of baicalein is formed at the 144 h time point.

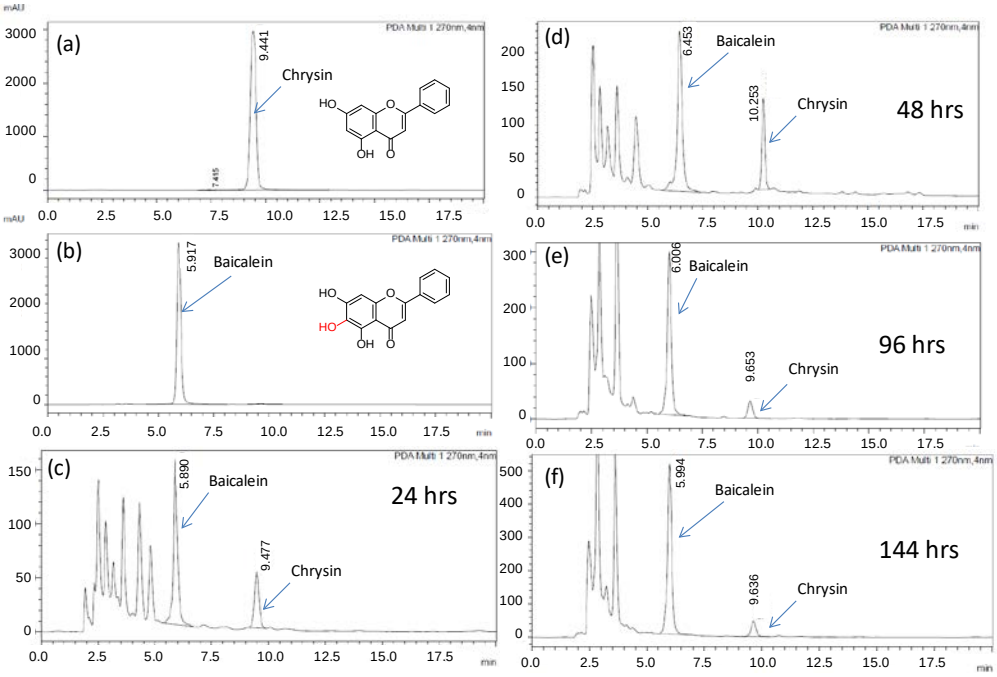
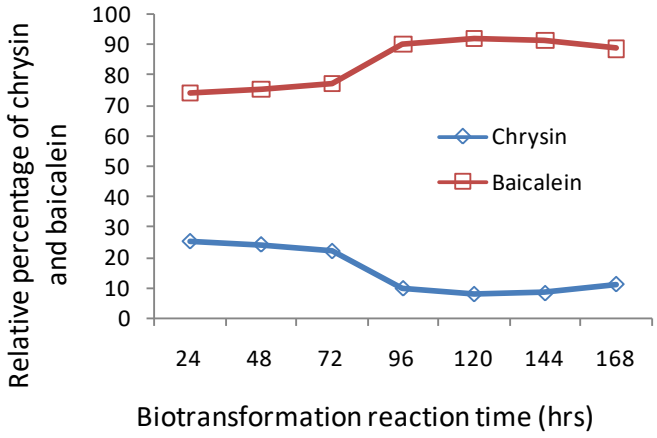


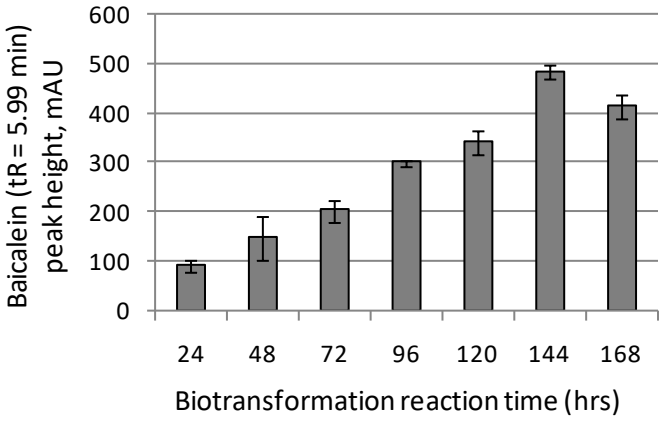
Figure 6.2. HPLC analysis of biotransformation reaction at different time intervals. The concentration of sample injected is kept constant at each time interval; therefore there is an increased AUC (and peak height) as the time increases from 24 to 144 hrs.

The relative percentages (AUC) of chrysin and baicalein at different time intervals are depicted in Figure 6.3a. As shown in Figure 6.3a, the percentage conversion of chrysin to baicalein increased with time. Similarly, Figure 3b shows increased peak height (mAU) of baicalein with increase in reaction time. Figures 6.3a and 6.3b, in combination, indicate

that 144 h is the optimal reaction time for this transformation. Further details of HPLC analysis are provided in supporting information, section S5 (Supporting Information).



(a)



(b)

Figure 6.3. (a) Relative percentage of chrysin and baicalein at different time intervals during a typical biotransformation reaction (the percentages are based on the AUC of the peaks in HPLC analysis at 270 nm). (b) Baicalein (tR = 5.99 min) peak height at different time intervals during biotransformation reaction.

Isolation and characterization of baicalein. Initial isolation attempts using normal phase silica gel column chromatography showed significant loss in product yield. Therefore, we attempted reverse phase C18 silica gel column chromatography where water-methanol was used as the mobile phase. The product was isolated at 50% methanol in water and was characterized by spectral analysis and by comparison with reference sample.

The ^{1}H NMR of chrysin contains a typical bunch of three singlets at chemical shift values in the range of 6 to 7 ppm. These three singlets at δ 6.24, 6.49 and 6.74 ppm correspond to the protons present at C6, C8 and C3 positions. The C6-proton appears with an up-field shift in comparison to two other aromatic protons at C3 and C-6, because of the shielding effect from two adjacent C5 and C7 hydroxyls. It is obvious that the hydroxylation reaction is possible, either on A or C ring. Since there was no change in the chemical shift value pattern of C ring (d 7.91, dd, 2H and d 7.48, m, 3H), it would indicate that the C-ring is intact and no hydroxylation had taken place on this ring. The hydroxylation on A ring has two possibilities, either C6- or C8 hydroxylation. Hydroxylation at C6-position will form baicalein (5,6,7-trihydroxyflavone) whereas hydroxylation at C8-position will form norwogonin (5,7,8-trihydroxyflavone) as a product. It was interesting to see that, in the ^{1}H NMR of the obtained product, the up-field singlet (δ 6.24 ppm) disappeared. This singlet peak in chrysin corresponds to the proton present at C6-position. This gave us a clear indication that hydroxylation occurred at the C6-position, which means that the product is 5,6,7-trihydroxyflavone. This is the naturally occurring flavone, commonly named as ‘baicalein’. Furthermore, on comparison of the ^{1}H NMR of the obtained product with norwogonin,[35] the possibility of norwogonin as the product was ruled out. The biotransformation of chrysin using natural yeast, *Rhodotorula glutinis* yielded C-8

hydroxylated product norwogonin,(Sordon et al., 2015) however in this present study, C-6 hydroxylated product (baicalein) was formed, which may be possibly because of the regio-specificity of the CYP1A1 enzyme.

Mass analysis of the isolated product showed *m/z* peak at 271 in ES+ve mode, which matched with the predicted product. The final confirmation of the assigned product was done by co-TLC and HPLC analysis with the reference standard of baicalein (CAS number: 491-67-8)' purchased from Sigma-Aldrich. TLC images as well as HPLC analysis clearly matched the reference standard (TLC images are shown in Supporting Information -S3).

The LC-MS analysis was also performed for the extract as well as isolated baicalein (Figure 6.4).

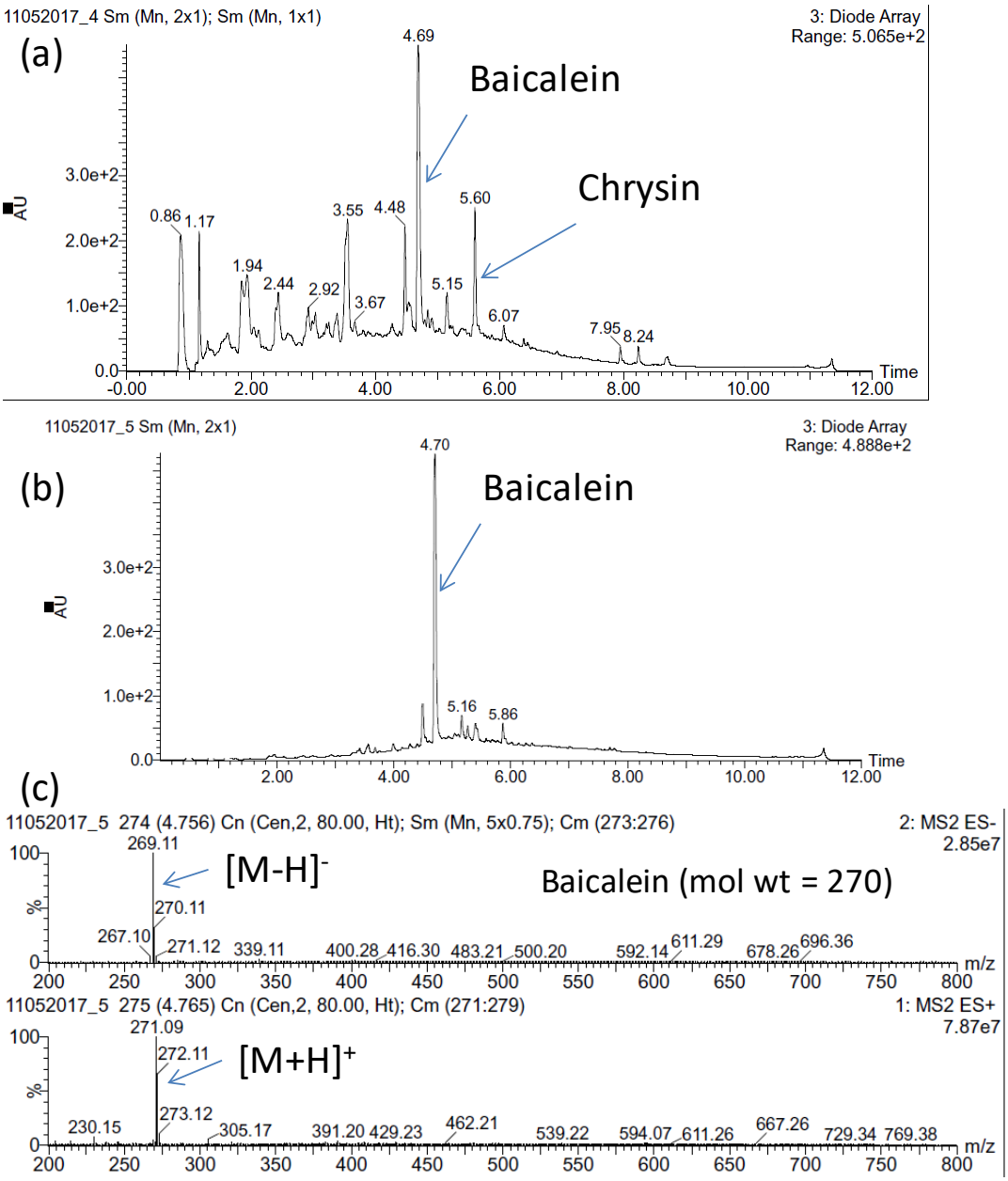


Figure 6.4. LC-MS analysis of reaction mixture at 144 h. (a) LC chromatogram of reaction mixture after 144 h of incubation. (B) LC chromatogram of isolated product; (c) Mass spectra of peak at tR 4.69 min.

Docking of chrysin with CYP1A1. In order to decipher the rationale for regio-selective hydroxylation, chrysin was docked with the substrate binding site of CYP1A1 enzyme (PDB ID: 4I8V). The interactions of chrysin with CYP1A1 are depicted in Figure 6.5. It is interesting to note that the A-ring of chrysin orients towards the heme. Furthermore,

the C-6 carbon of A-ring is present in close-proximity with heme protein, suggesting that the reactive heme-oxo intermediate should possibly form at this position. Other key interactions which help in stabilizing this orientation of chrysin includes: (a) hydrophobic π-π interactions of Phe-224 of I-helix with B and C rings; (b) π-π interactions of Phe-319 with A ring; and (c) polar H-bonding of C-7 hydroxyl group with Ser-122. This observed orientation, excludes the possibility of hydroxylation at C-8 and at aromatic CH of B and C rings.

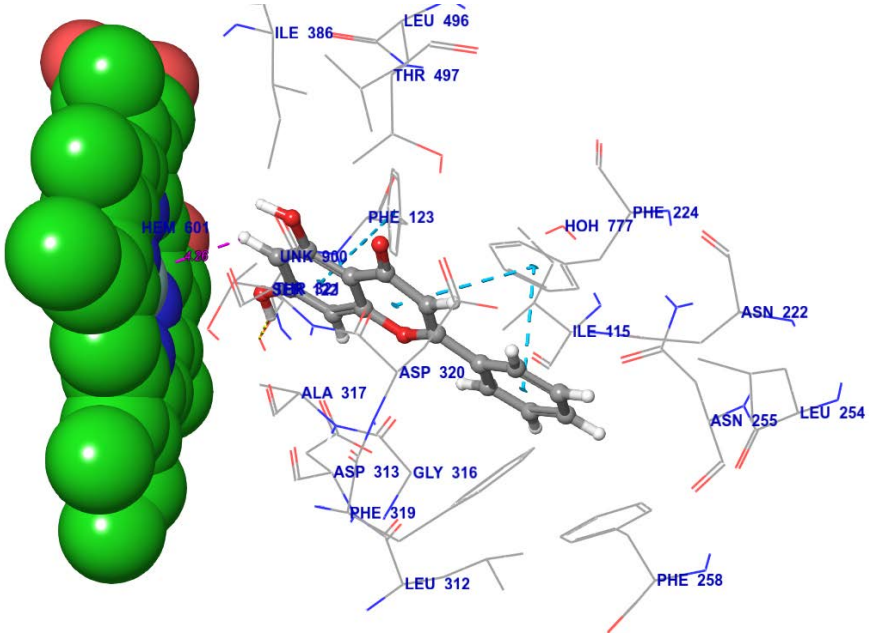


Figure 6.5. Molecular docking of chrysin with CYP1A1, showing the predicted site of hydroxylation as C6.

Our efforts using yeast whole cells have resulted in the development of a reproducible preparative-scale biotransformation process for the conversion of chrysin to baicalein (5,6,7-trihydroxyflavone). According to the literature, the medicinal effects of baicalein are more profound than that of chrysin. Furthermore, commercially available baicalein is at least 60-times more expensive than chrysin. Thus, this protocol described here can be

utilized for production of a high value phytochemical from a low value one, using a simple, low-cost, one-step biotransformation reaction.

In conclusion, we have demonstrated the ability of whole yeast cells, that overexpress the human CYP1A1 enzyme, to catalyse biotransformation of >92% of the natural flavonoid chrysin to baicalein. Optimal aeration, neutral pH and maintenance of glucose concentration, throughout the reaction, played very important roles in the biotransformation reaction. The example demonstrated in this paper, provides an opportunity for further exploring the utility of stable recombinant CYP enzyme-expressing yeast cells for industrial production of medicinally important polyphenolic compounds.

6.3.5 Supporting information (SI)

S1. CYP activities in yeast cell culture continue to increase with time.

Results in Figure S1.1 show a remarkable increase of cell numbers, over 72 h, of a 100 ml yeast culture grown in YPD, in a 500 ml baffled shake flask, of the yeast strain [W303-1a::CYP1A1(HIS3^{+}), CYP1A1(URA3^{+})] that contains 2 copies of the human *CYP1A1* gene; it is likely that these results will be replicated in a fed-batch fermentor over a longer period of time. Similar results were obtained with the yeast strain [W303-1a::CYP1A2(HIS3^{+}), CYP1A2(URA3^{+})] that expresses 2 copies of the human *CYP1A2* gene.

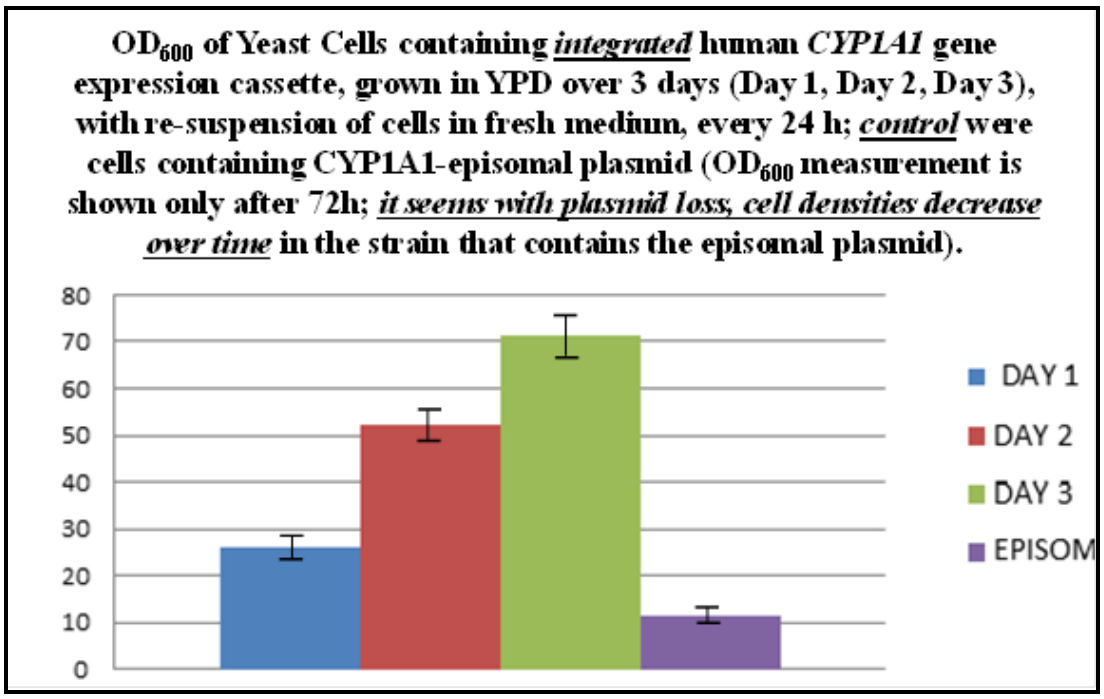


Figure S1.1. Growth, over 72 h, of the CYP1A1 yeast strain W303-1a::CYP1A1(HIS3$^+$), CYP1A1(URA3$^+$), replenished with fresh medium every 24 h. Results clearly show the 'stability' of growth of recombinant cells, over time, that contain integrated copies of the *CYP1A1* gene compared to the strain that bears a 2-micron based episomal plasmid (labelled 'EPISOM'). The y-axis represents optical density measured at 600 nm (OD_{600}). The values represent mean and standard deviations from three independent experiments.

Results Figure S1.2 show a gradual increase of cellular CYP1A1 activity, over time (24 h ➔ 72 h), which may also be replicated in a fed-batch fermentor over a longer period of time.

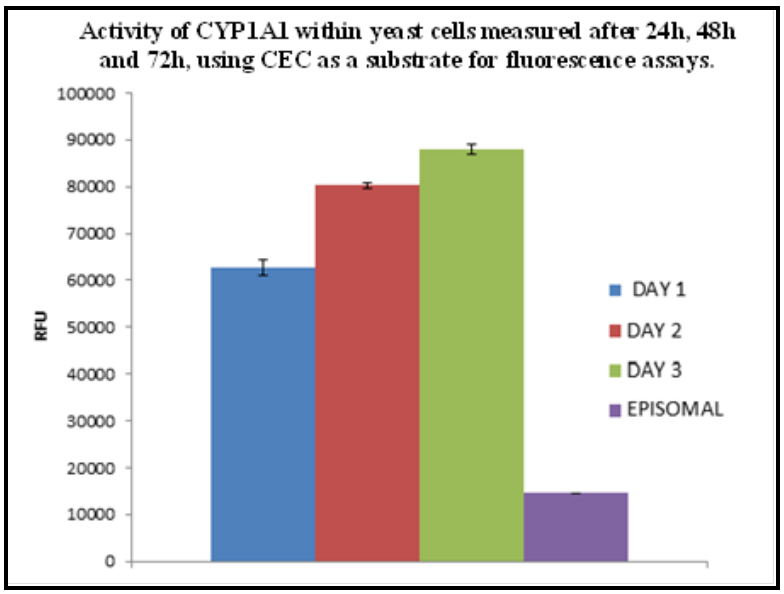


Figure S1.2. Activity of the 2-copy CYP1A1 yeast strain; CYP enzyme activity was measured every 24 h using the EROD assay.[1-3] The y-axis represents relative fluorescence units (RFUs). The values represent mean and standard deviations from three independent experiments.

Results confirm a gradual increase of cellular CYP1A1 activity in cells cultured over a period of time (24 h ➔ 72 h). Similar results were obtained with the yeast strain [W303-1a::CYP1A2(HIS3^{+}), CYP1A2(URA3^{+})] that expresses 2 copies of the human *CYP1A2* gene.

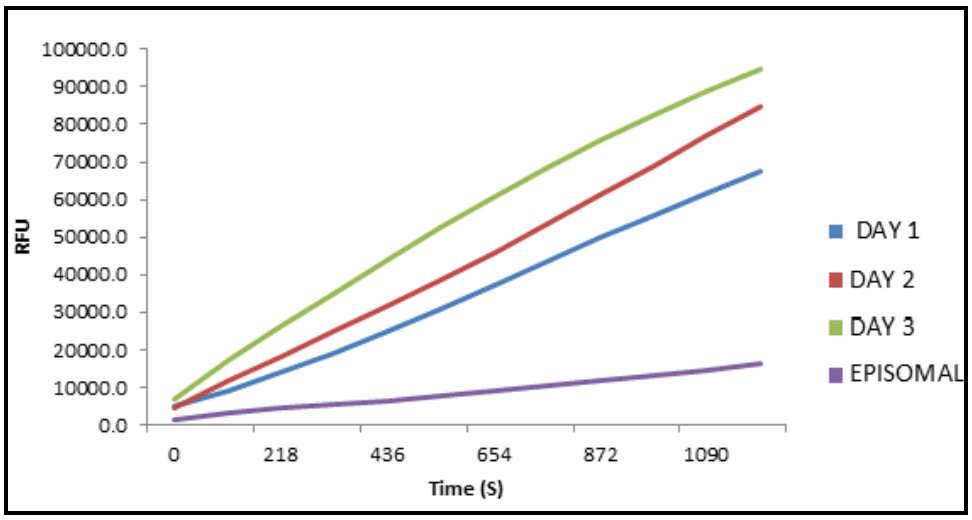


Figure S1.3. Kinetics of CYP1A1 activities, as shown in Figure S 1.2, measured via the EROD assay.[1-3] The y-axis represents relative fluorescence units (RFUs). The values represent mean and standard deviations from three independent experiments.

S2. Maps of human *CYP1A1* and *CYP1A2* gene bearing plasmids that were integrated into the chromosomal loci of the *HIS3* (chromosome XV) and *URA3* (chromosome V) genes in the yeast strain W303-1a.

Chromosomal integration was carried out through homologous recombination in the yeast strain W303-1a (ATCC 208352), following standard yeast transformation protocols.

The human *CYP1A1* and *CYP1A2* gene sequences were isolated from a human liver cDNA library (BioCat). The *ADH2* promoter (ADH2p) and the *SUC2* terminator (SUC2t) sequences were isolated from genomic DNA obtained from the strain S228C (ATCC 204508). Isolation of all genetic sequences was performed via the polymerase chain reaction (PCR), using gene specific primers.

Figure S2.1 shows the map of the human *CYP1A1* gene encoding plasmid that allows integration into the strain W303-1aat the chromosomal locus where the *HIS3* gene lies. The map shows common restriction sites that occur in the plasmid only once.

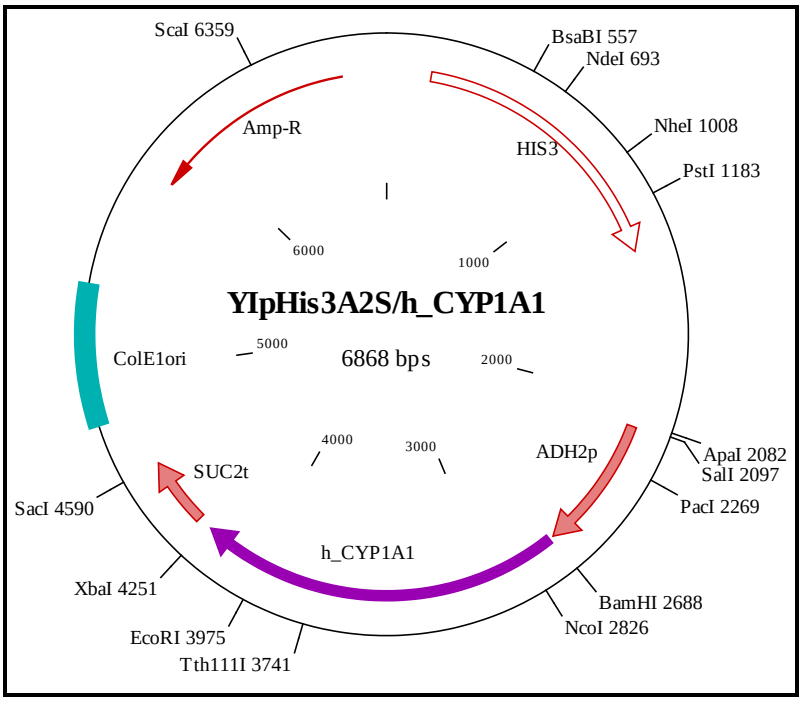


Figure S2.1. Human *CYP1A1* gene encoding *HIS3* integrating plasmid.The plasmid was linearized at the *Nde*I restriction site for integration at the *HIS3* chromosomal locus of strain W303-1a to obtain the strain W303-1a::CYP1A1(HIS3^{+}).

Figure S2.2 shows the map of the human *CYP1A1* gene encoding plasmid that allows integration into the strain W303-1a at the chromosomal locus where the *URA3* gene lies. The map shows common restriction sites that occur in the plasmid only once.

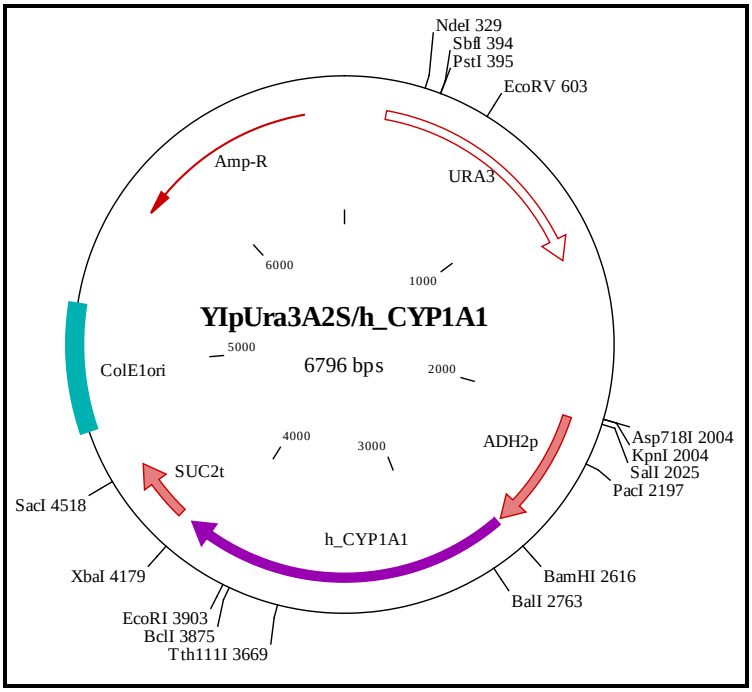


Figure S2.2. Human *CYP1A1* gene encoding *URA3* integrating plasmid.The plasmid was linearized at the *EcoR*V restriction site for integration at the *URA3* chromosomal locus of strain W303-1a::CYP1A1(HIS3^{+}) to obtain the strain W303-1a::CYP1A1(HIS3^{+}), CYP1A1(URA3^{+}) which was used primarily for the biotransformation of chrysin to baicalien, and for optimization of the biotransformation process.

Figure S2.3 shows the map of the human *CYP1A2* gene encoding plasmid that allows integration into the strain W303-1a at the chromosomal locus where the *HIS3* gene lies. The map shows common restriction sites that occur in the plasmid only once.

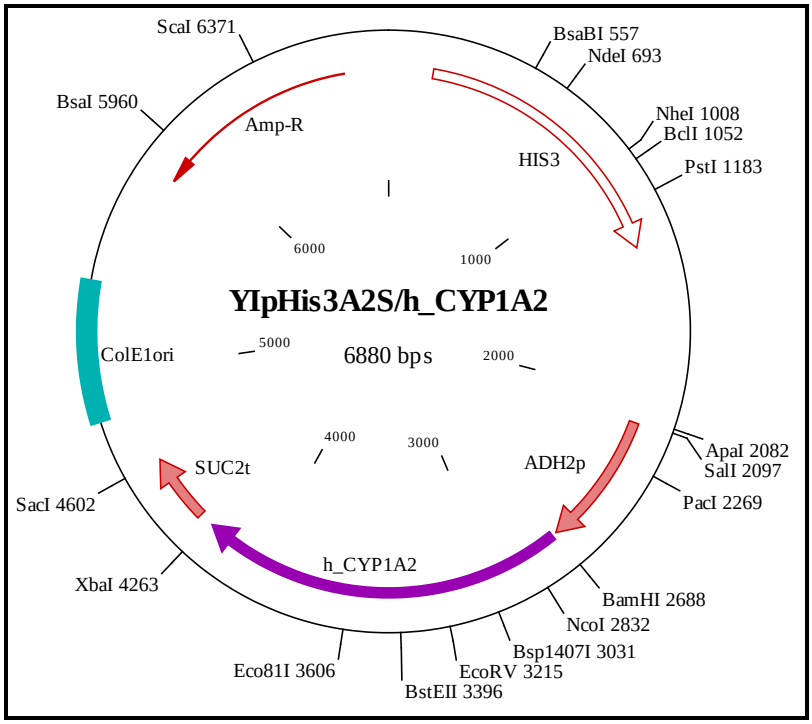


Figure S2.3. Human *CYP1A2* gene encoding *HIS3* integrating plasmid. The plasmid was linearized at the *Nde*I restriction site for integration at the *HIS3* chromosomal locus of strain W303-1a to obtain the strain W303-1a::CYP1A2(HIS3^{+}).

Figure S2.4 shows the map of the human *CYP1A2* gene encoding plasmid that allows integration into the strain W303-1a at the chromosomal locus where the *URA3* gene lies. The map shows common restriction sites that occur in the plasmid only once.

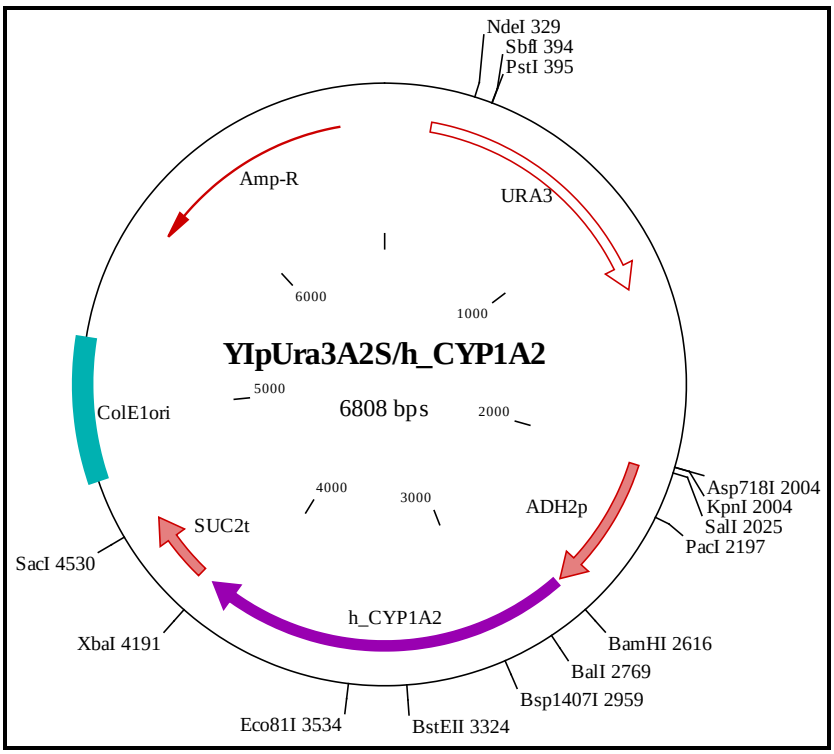
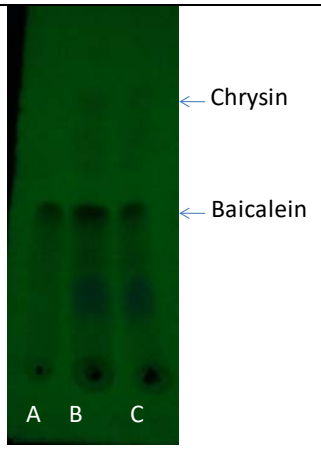
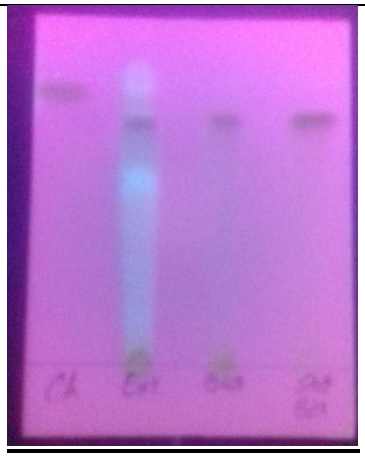
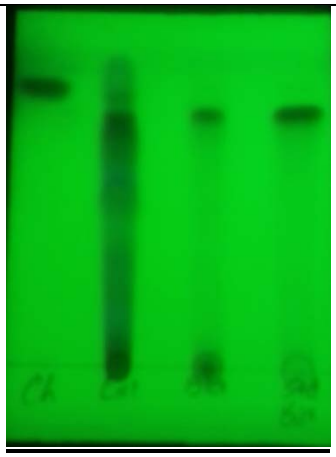


Figure S2.4. Human *CYP1A2* gene encoding *URA3* integrating plasmid. The plasmid was linearized at the *Pst*I restriction site for integration at the *URA3* chromosomal locus of strain W303-1a::CYP1A2(HIS3) to obtain the strain W303-1a::CYP1A2(HIS3^{+}), CYP1A2(URA3^{+}) which was used for the initial biotransformation of chrysin to baicalien.

S3. TLC profile of Chrysin to Baicalein conversion

A

		
A **Spots from left to right:** A. Isolated baicalein B. co-spot (A+C) C. Reaction mixture **Mobile Phase:** 3% MeOH in DCM Detection wavelength: 254 nm	**B** **Spots from left to right:** • Chrysin • reaction mixture • isolated baicalein and • reference standard of baicalein **Mobile Phase:** 40% EtOAc: Hexane (with 0.1% acetic acid) Detection wavelength: 254 nm	**C** **Spots from left to right:** • Chrysin • reaction mixture • isolated baicalein and • reference standard of baicalein **Mobile Phase:** 40% EtOAc: Hexane (with 0.1% acetic acid) Detection wavelength: 365 nm

S4. Western blots of human CYP1A1 and CYP1A2 proteins expressed in baker's yeast.

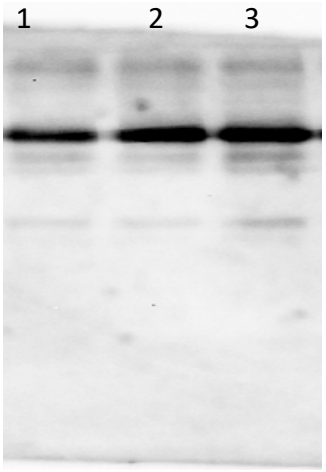


Figure S4.1.Western blot of CYP1A1 protein. 10^6 cells from each recombinant yeast strain were from a 24 h SD minimal medium culture. They were lysed with glass beads in a SDS containing buffer which is routinely used for yeast cell lysis; 15 µl from each 100 µl supernatant was loaded in lanes 1 and 2. Lane 1, cells from strain W303-1a::CYP1A1(HIS3$^+$) which expresses 1 copy of *CYP1A1* gene; Lane 2, cells from strain W303-1a::CYP1A1(HIS3$^+$), CYP1A1(URA3$^+$) which expresses 2 copies of *CYP1A1* gene; Lane 3, 1.5 pmole of CYP1A1 microsomal enzyme isolated from whole yeast cells.

Figure S4.2.Western blot of CYP1A2 protein. Lane 1, 10^6 cells from the yeast strain W303-1a::CYP1A2(HIS3$^+$), CYP1A2(URA3$^+$), from a 24 h SD minimal medium culture, were lysed with glass beads in a SDS containing buffer which is routinely used for yeast cell lysis; 15 µl from 100 µl supernatant was loaded. Lane 2, 1 pmole of CYP1A2 microsomal enzyme obtained from Corning-Gentest (Catalogue #456203).

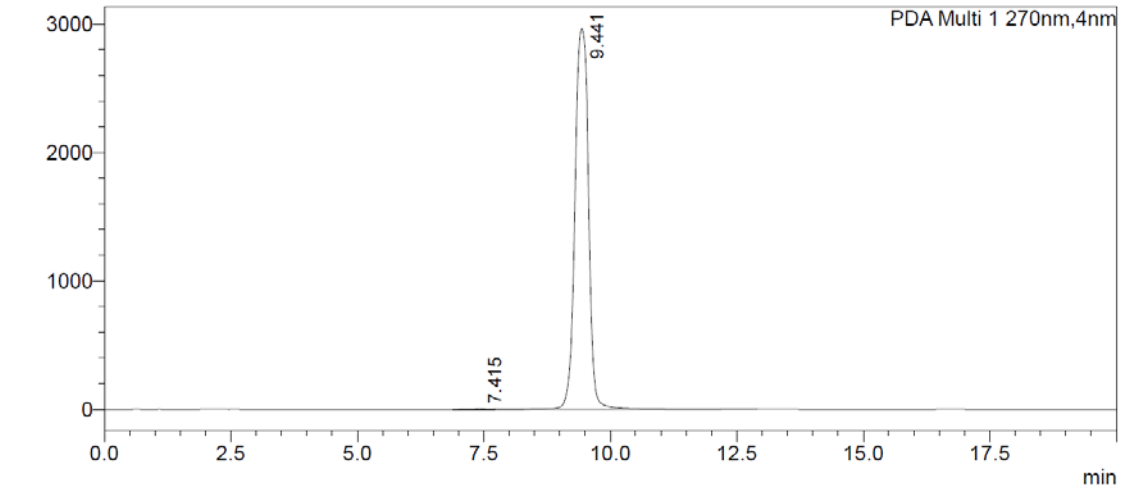
S5. HPLC additional details

Method: HPLC analysis was performed on Shimadzu LC-6AD system connected with C18 column (4.6 x 25 mm, 5 µ). Mobile phase consisted of A: 0.1% formic acid and B: methanol using isocratic elution (30: 70 – A: B). Flow rate was 1 mL/min. Detection wavelength was 270 nm.

HPLC spectras of reference standards of chrysin and baicalein in this method:

Chrysin

<Chromatogram>



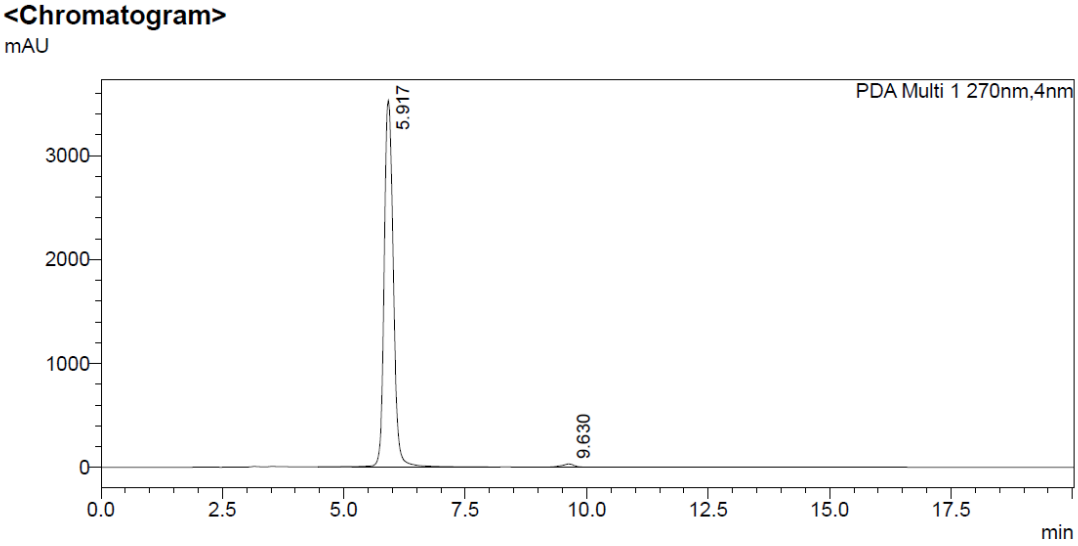
<Peak Table>

PDA Ch1 270nm

Peak#	Ret. Time	Area	Area%
1	7.415	63680	0.115
2	9.441	55469372	99.885
Total		55533052	100.000

Baicalein:

<Chromatogram>



<Peak Table>

PDA Ch1 270nm

Peak#	Ret. Time	Area	Area%
1	5.917	46350302	98.772
2	9.630	576173	1.228
Total		46926475	100.000

The analysis of HPLC data for biotrasformation reactions monitored at different time intervals is provided below. The analysis was performed at 270 nm (λ_{max} of baicalein)

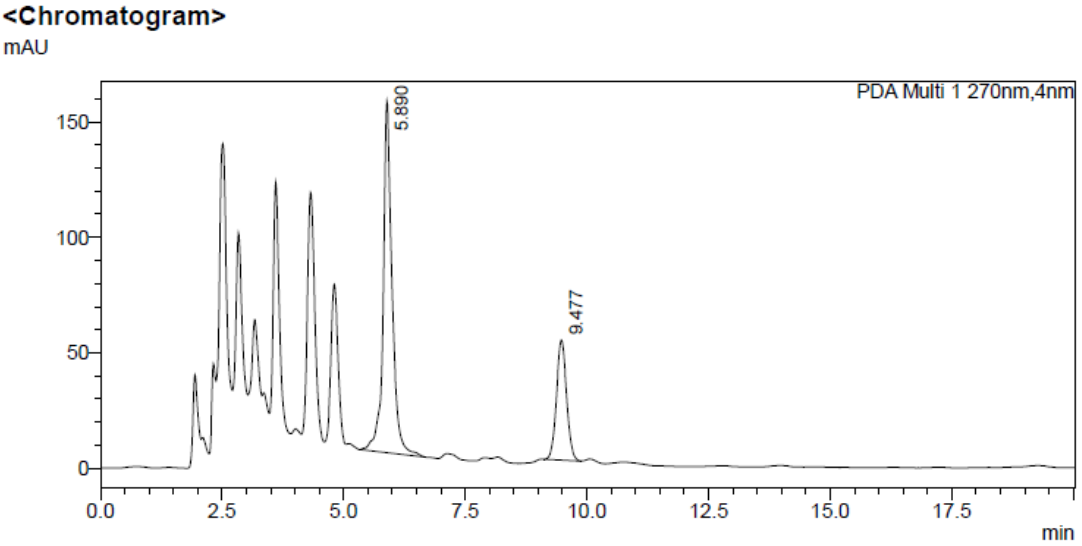
Time (hrs)	**Peak area (AUC) percentage[a]**			Peak height of baicalein (mAU)	Relative ratio of baicalein to chrysin
	A bunch of unknown peaks at tR = 2-5 min	Baicalein (tR = 5.99 min)	Chrysin (tR = 9.63 min)		
24	77	16	7	90.7	74: 26
48	77	21	2	148.8	75: 25
72	70	19	11	204	78: 22
96	76	21	3	300	90:10
120	76	22	2	342	92: 8
144	72	**26**	2	484	**92: 8**
168	81	17	2	414	89:11

[a]Total area percentage = 100

The concentration of sample injected is kept constant at each time interval; therefore there is an increased AUC (and peak height) as the time increases from 24 to 144 hrs. HPLC chromatograms at different time points are shown below.

24 hrs

<Chromatogram>



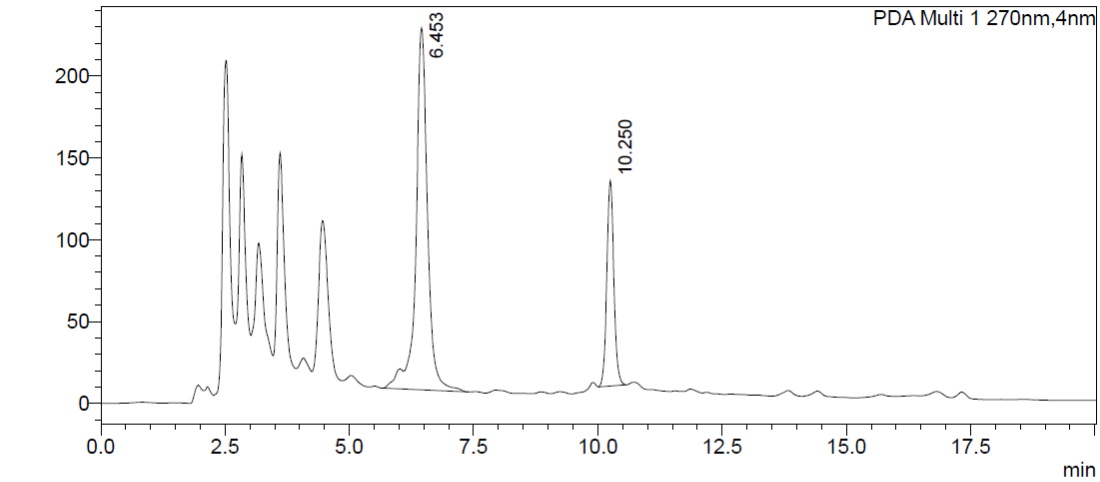
<Peak Table>

PDA Ch1 270nm

Peak#	Ret. Time	Area	Area%
1	5.890	1930895	72.077
2	9.477	748026	27.923
Total		2678921	100.000

48 hrs

<Chromatogram>



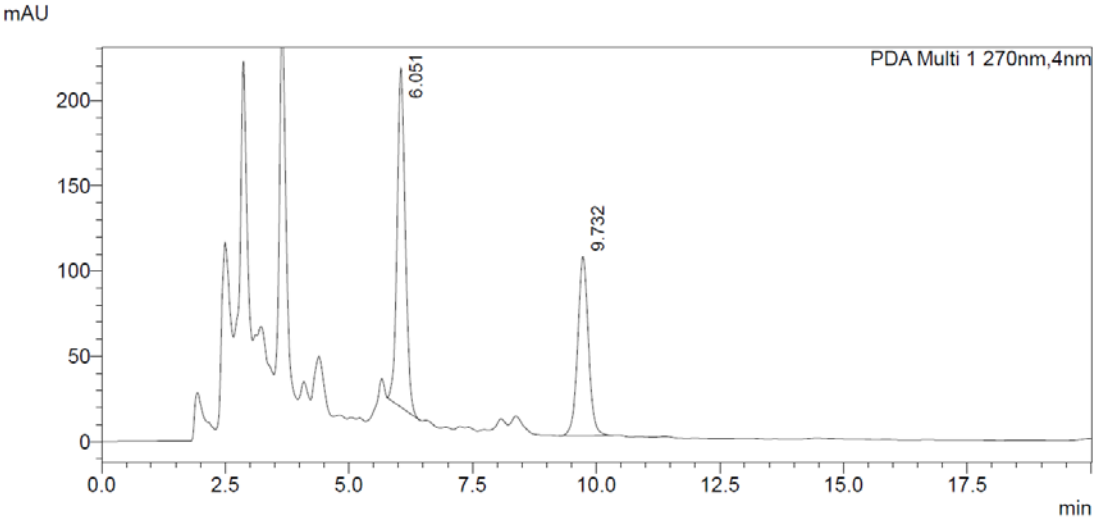
<Peak Table>

PDA Ch1 270nm

Peak#	Ret. Time	Area	Area%
1	6.453	3609769	74.514
2	10.250	1234675	25.486
Total		4844444	100.000

72 hrs

<Chromatogram>



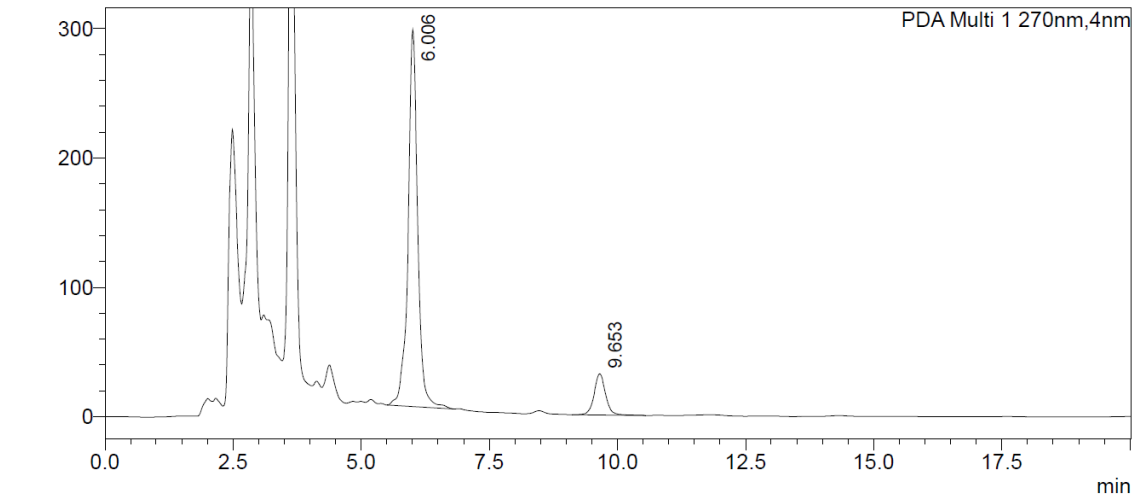
<Peak Table>

PDA Ch1 270nm

Peak#	Ret. Time	Area	Area%
1	6.051	2272461	58.973
2	9.732	1580907	41.027
Total		3853368	100.000

96 hrs

<Chromatogram>



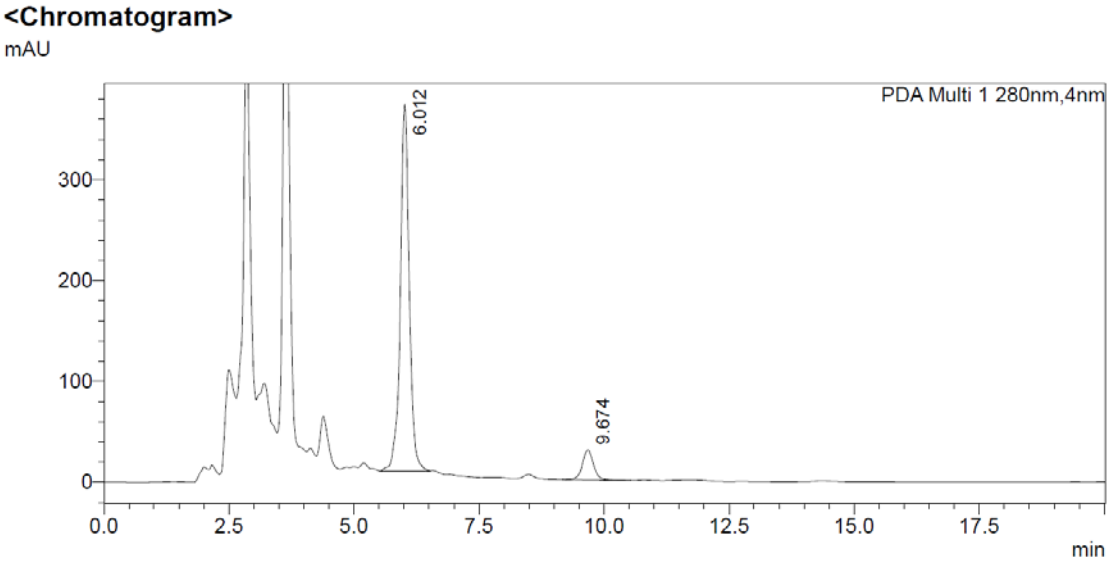
<Peak Table>

PDA Ch1 270nm

Peak#	Ret. Time	Area	Area%
1	6.006	3789934	88.529
2	9.653	491077	11.471
Total		4281011	100.000

120 hrs

<Chromatogram>



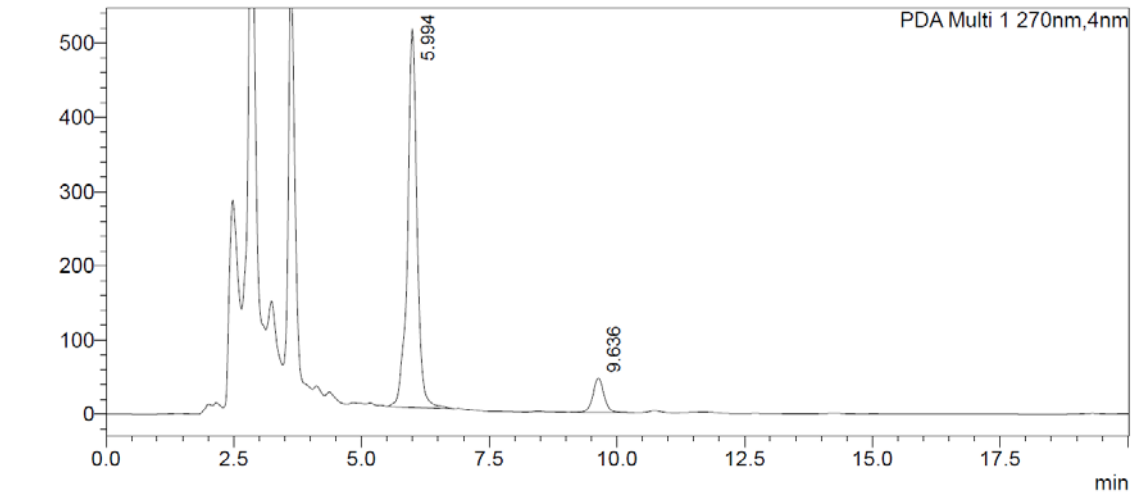
<Peak Table>

PDA Ch1 280nm

Peak#	Ret. Time	Area	Area%
1	6.012	4506166	90.766
2	9.674	458454	9.234
Total		4964620	100.000

144 hrs

<Chromatogram>



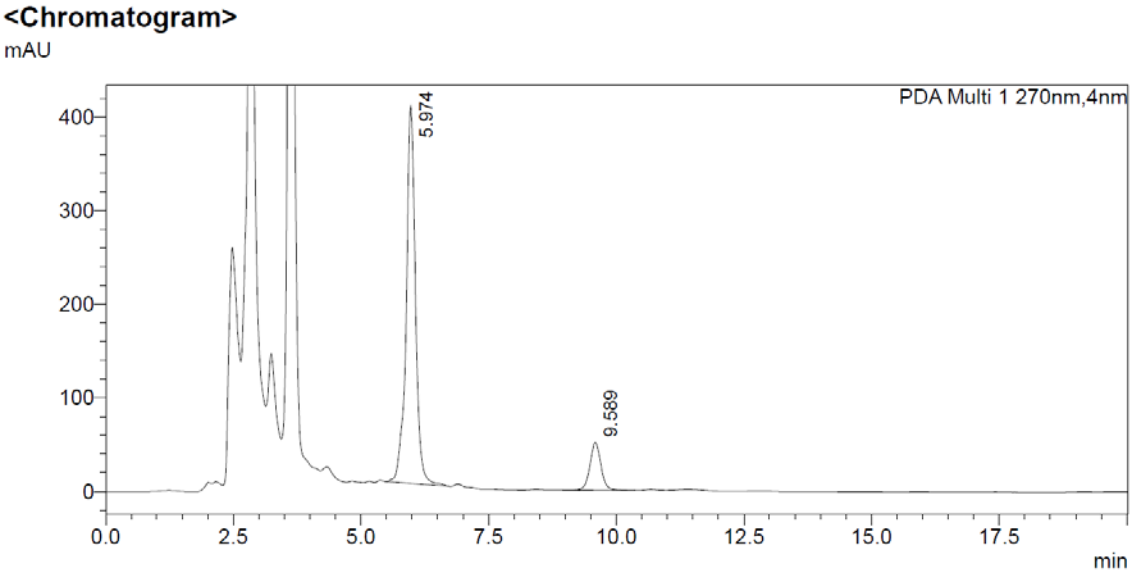
<Peak Table>

PDA Ch1 270nm

Peak#	Ret. Time	Area	Area%
1	5.994	6591521	90.395
2	9.636	700393	9.605
Total		7291913	100.000

168 hrs

<Chromatogram>

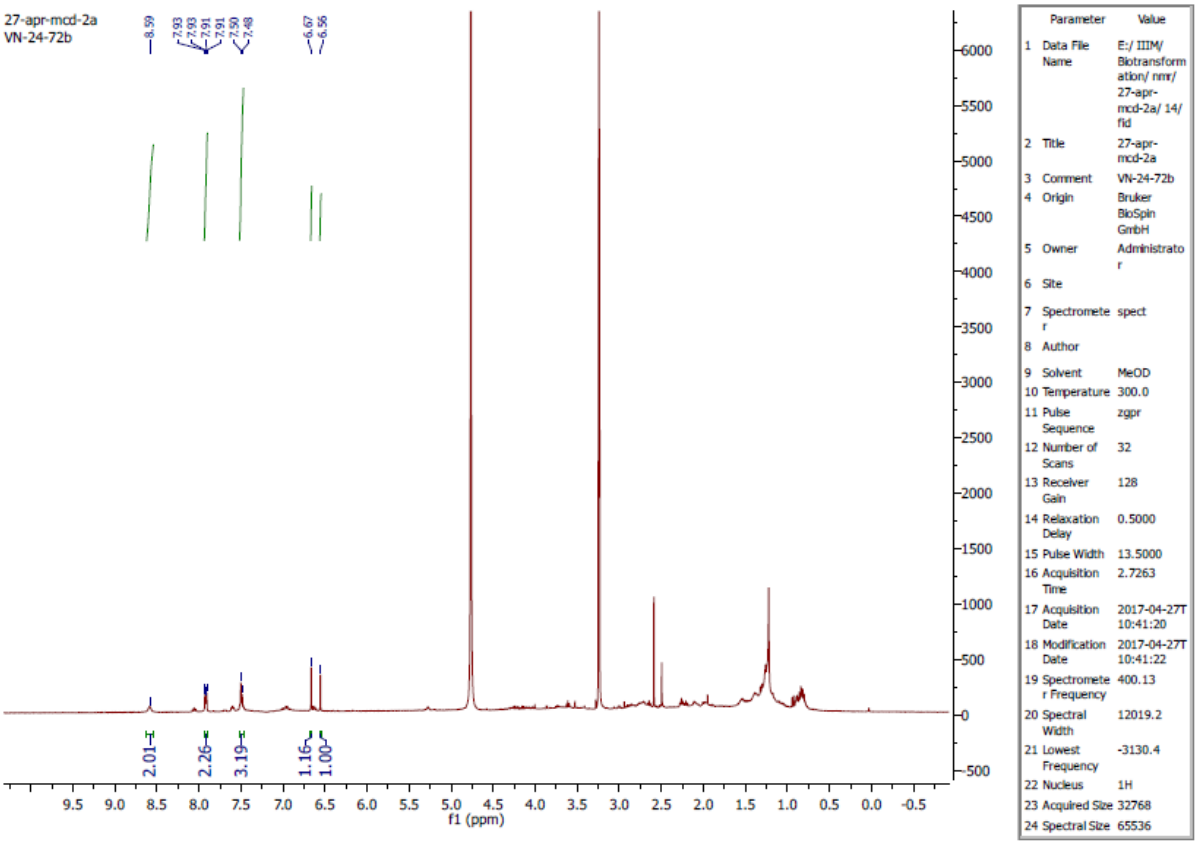


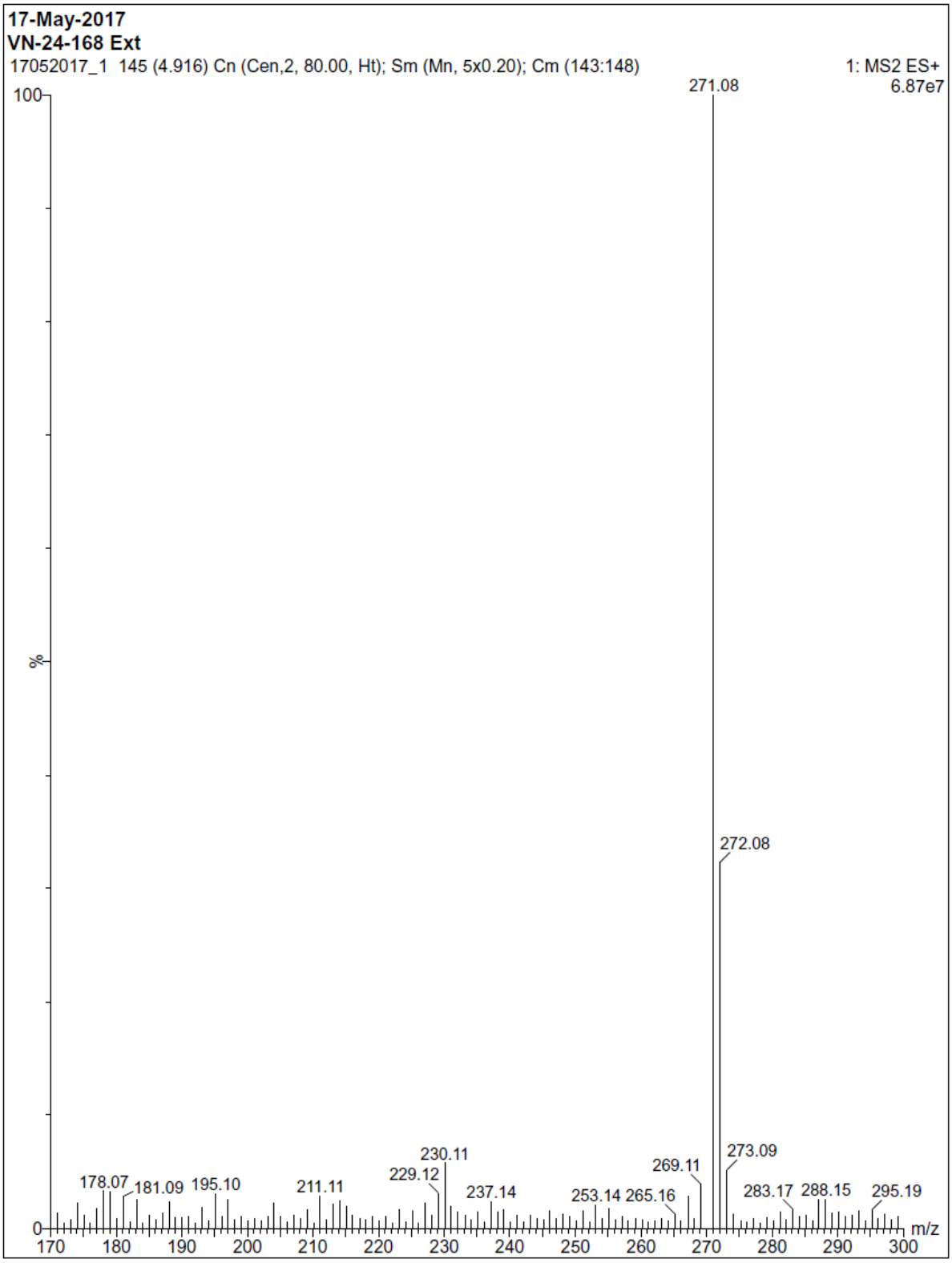
<Peak Table>

PDA Ch1 270nm

Peak#	Ret. Time	Area	Area%
1	5.974	5097191	86.912
2	9.589	767558	13.088
Total		5864749	100.000

S6. NMR data (Solvent: CD_3OD) and MS spectra scan of the isolated product (baicalein)

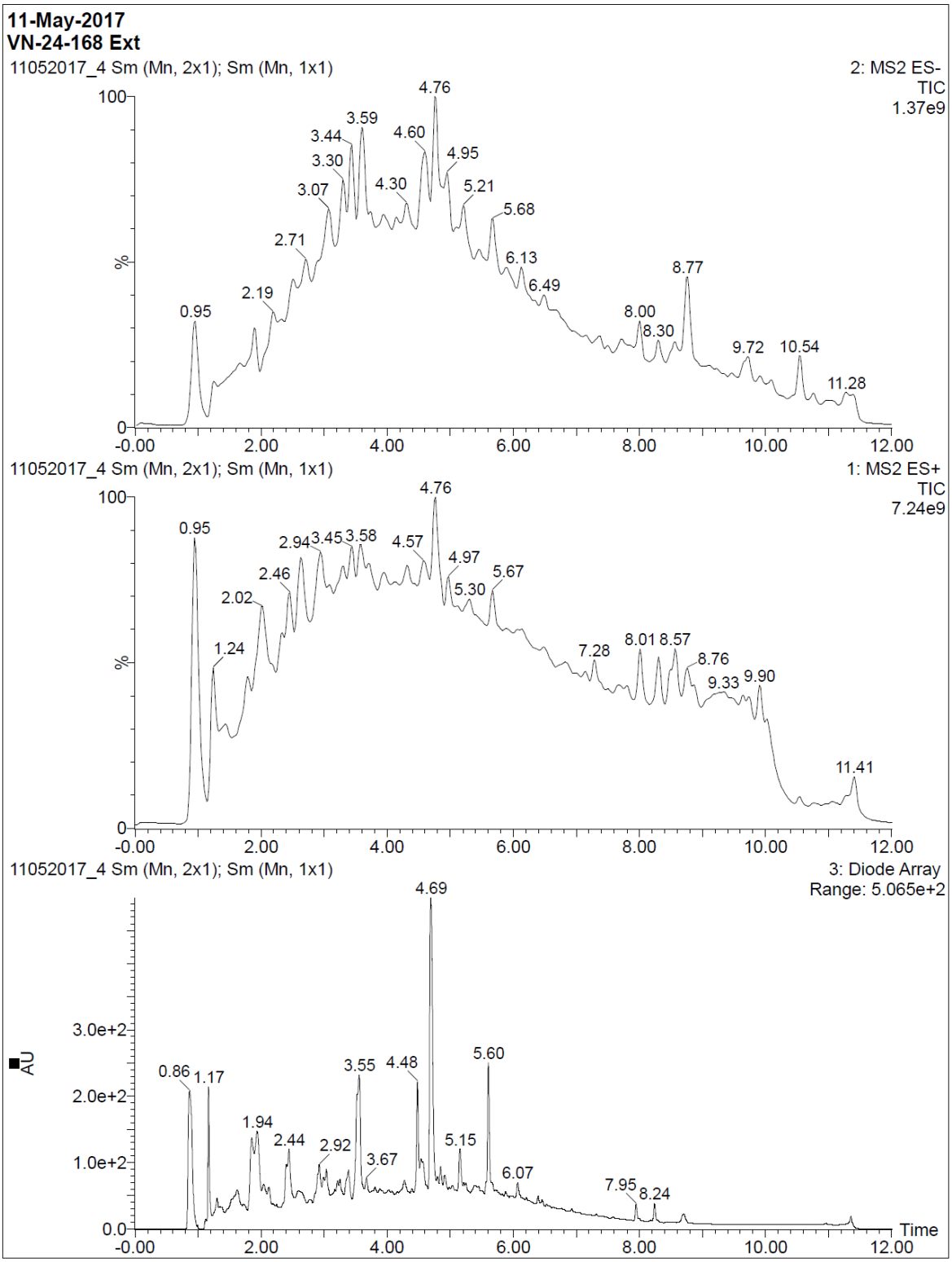



17-May-2017
VN-24-168 Ext
17052017_1 145 (4.916) Cn (Cen,2, 80.00, Ht); Sm (Mn, 5x0.20); Cm (143:148)
1: MS2 ES+
6.87e7
100
%
0
271.08
272.08
273.09
230.11
229.12
269.11
178.07
181.09
195.10
211.11
237.14
253.14
265.16
283.17
288.15
295.19
170
180
190
200
210
220
230
240
250
260
270
280
290
300
m/z

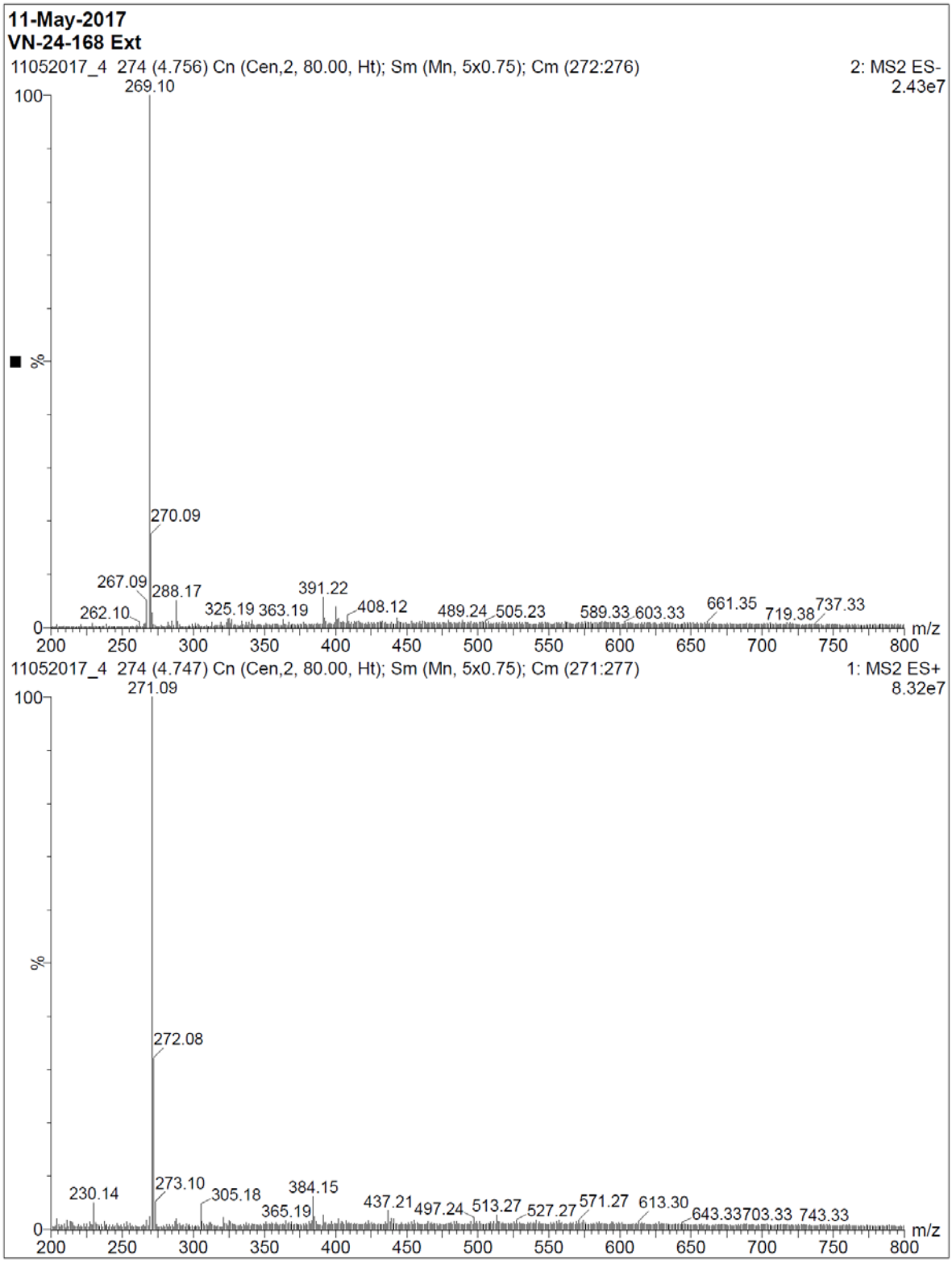
S7. LCMS analysis of biotransformation reaction.

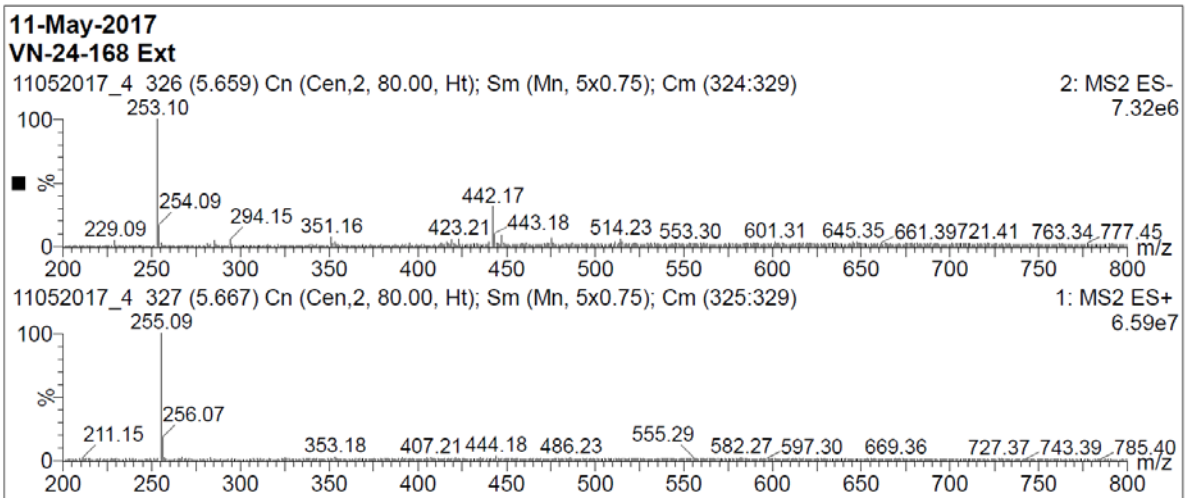
Method: LC-MS analysis was performed on Waters Acquity UPLC. The column used was C18, 1.7 µ with dimensions of 100 x 2.1 mm (column temp. 30 °C). Binary gradient system was used. Mobile phase A consisted of 5% acetonitrile in water with 0.1% formic acid. Mobile phase B consisted of acetonitrile with 0.1% formic acid. Gradient details are: Time in min (% B concentration): 0.01 (10), 0.25 (10), 9.00 (100), 10.00 (100), 11.00 (10), 12.00 (10). PDA range: 220 nm to 400 nm; flow rate: 0.3 mL/min.

The LCMS data of biotrasformation reaction, at 168 hrs incubation time: The LC and TIC profile is shown below.



The MS spectra of 4.69 min (baicalein, mw = 270) and 5.60 min (chrysin, mw = 254) peaks




11-May-2017
VN-24-168 Ext
11052017_4 326 (5.659) Cn (Cen,2, 80.00, Ht); Sm (Mn, 5x0.75); Cm (324:329)
2: MS2 ES-
7.32e6
253.10
254.09
294.15
229.09
351.16
442.17
423.21
443.18
514.23
553.30
601.31
645.35
661.39
721.41
763.34
777.45
m/z
11052017_4 327 (5.667) Cn (Cen,2, 80.00, Ht); Sm (Mn, 5x0.75); Cm (325:329)
1: MS2 ES+
6.59e7
255.09
256.07
211.15
353.18
407.21
444.18
486.23
555.29
582.27
597.30
669.36
727.37
743.39
785.40
m/z

6.4 Biotransformation of codeine to morphine using whole yeast cells expressing human CYP2D6 variants, – revelation of CYP2D6 variant P34S/A122V/S486T being a far superior metaboliser of codeine than the wild-type (Met^{374}) and Val^{374} variants

(Manuscript in preparation: Ibidapo S Williams et al; will be published as soon as molecular modelling is complete].

6.4.1 Abstract

CYP2D6 belongs to the cytochrome P450 (CYP) family of enzymes and is known to metabolise codeine to morphine within the human body. It has been reported that, in general, only up to 15% of codeine undergoes *O*-demethylation to form morphine within humans. Morphine is codeine's most active metabolite and is 200-fold stronger than codeine that induces which act as an agonist of the pain mediator, the μ-opioid receptor. I have established a platform technology that allows stable expression, in baker's yeast, of human CYP genes from yeast's chromosomal loci (Chapter 5). Three CYP2D6 variant genes, 2D6-1 (NCBI Accession No NM_000106; M374V or Val^{374}), 2D6-2 (NCBI Accession No M20403; wild type or Met^{374}) and 2D6-C (isolated from a liver cDNA library), were cloned in yeast integration vectors for expression within live yeast cells. Surprisingly, results show that the variant 2D6-C (P34S/A122V/S486T), which has never been reported before, yields >70% of morphine whereas the yields for 2D6-1 and 2D6-2 were around only 30%. It seems that the variants 2D6-1 and 2D6-2 predominantly form norcodeine, which involves *N*-demethylation of codeine, with >55% yield. All reactions

were carried out in baffled shake-flasks under conditions that mimic fed-batch fermentation. The specificity of 2D6-C for *O*-demethylation and of 2D6-1 and 2D6-2 for *N*-demethylation is being explored via molecular modelling. The system described here, on further optimisation, could find generic use in high-yield chemical reactions for the formation of regio-specific de-alkylation products.

6.4.2 Introduction

Codeine is an opioid class of painkiller. The central nervous system and gastro intestinal system are the two major areas of the body where codeine exerts its effects. Codeine's binding to the μ-opioid receptor is weak. However, morphine, a metabolite of codeine, has a greater than 200-fold affinity for the μ-opioid receptor than codeine. Codeine undergoes *O*-demethylation by CYP2D6 to form the metabolite, morphine. It has been reported that only up to 15% of codeine is metabolised to morphine and the remaining 85% is converted to inactive forms (codeine-6-glucuronide and nor-codeine via *N*-demethylation; Figure 6.6) and excreted. Morphine itself can undergo further metabolism to nor-morphine (again via *N*-demethylation; Figure 6.6) and morphine-6-glucuronide. Like codeine itself, glucuronides and *N*-de-methylated derivatives (Figure 6.6) of both codeine and morphine have also weak affinity for the μ-opioid receptor. Therefore, they cannot act as effective painkillers like morphine (Palo, 2012; Caudle et al., 2017).

Drug abuse and the use of illegal heroin (Figure 6.6), identified in blood samples, are persistent menace in our society right from the past till the present day. To assess the level of heroin in the blood system of users is not easy especially because the metabolite 6-acetylmorphine (6-AM; Figure 6.6) is difficult to assess due to its short half-life which

ranges up to a minute (Goldberger at al., 2005). Total codeine-to-morphine ratio is, therefore, employed to detect the presence and amount of heroin or codeine consumption (Sindrup et al., 1990).

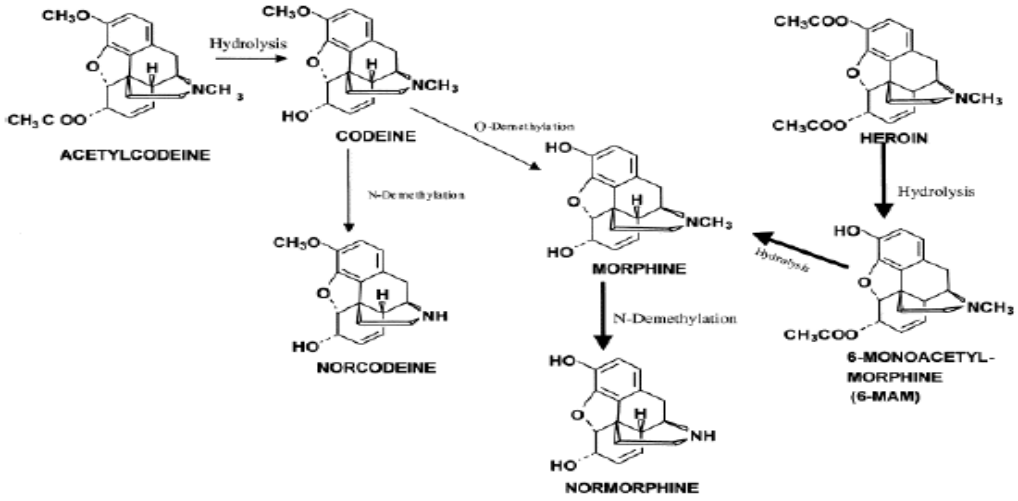


Figure 6.6. Codeine undergoes *O*-demethylation to form morphine and *N*-demethylation to form norcodeine. [C. Meadway et al., 2002].

CYP2D6 and its isoforms are well known metabolisers of codeine to morphine (Sindrup et al., 1990). Any individual suffering from extreme low plasma concentrations (i.e. lack) of CYP2D6 enzyme is referred to as a poor metabolizer. It has been reported that individuals carrying the genotype of the ultra-rapid CYP2D6 metabolisers are said to be 45-fold higher in their concentration of morphine, the *O*-de-methylated metabolite of codeine, than individuals with poor metabolism (Yu et al., 1997). There is variation in the percentage of individuals carrying the 'high metaboliser' genotype, from 5-10% in Southern European populations to 10-30% in Asian and North/ East African countries (Sachse et al., 1997; Bernal et al., 1999; Aklillu et al., 1996).

The current system of rating the levels of heroin detected in the blood maybe unfair on certain individuals. The current rating (i.e. codeine-to-morphine ratio) is set at value above one (1) which is considered to indicate the use of heroin (Pearce et al., 2008). Since the levels of CYP2D6 and its isoforms vary from one individual to another, individuals who are active CYP2D6 metabolizers will wrongfully be accused of heroin abuse.

Moreover, individuals with ultra-rapid metabolizer *CYP2D6* genotype of codeine are also endangered because of the persistent high amounts of morphine concentrations in their bodies. This may result in confusion, sleepiness and shallow breathing. Also, new babies can suffer the effect of high concentration of morphine through their mothers' breast milk (Antoine , 2012).

6.4.3 Aim of experiments with CYP2D6 variants

The aim was to establish if whole yeast cells, expressing different CYP2D6 variant enzymes, could be used to metabolize proficiently a compound "A" to product "B". The reason for choosing codeine as a substrate was to gain proof of principle.

6.4.4 Results

10 micromoles (µmoles) of codeine were incubated in baffled shake-flasks that contained liquid cultures of three different yeast strains: that expressed (1) CYP2D6-1, (2) CYP2D6-2, and (3) CYP2D6-C enzymes. The protocol followed was exactly as the one described in Section 6.3 of this Chapter. The product formed was extracted with organic solvents, the solvents were evaporated and the residues were analysed using high-performance liquid chromatography (HPLC; Figure 6.7).

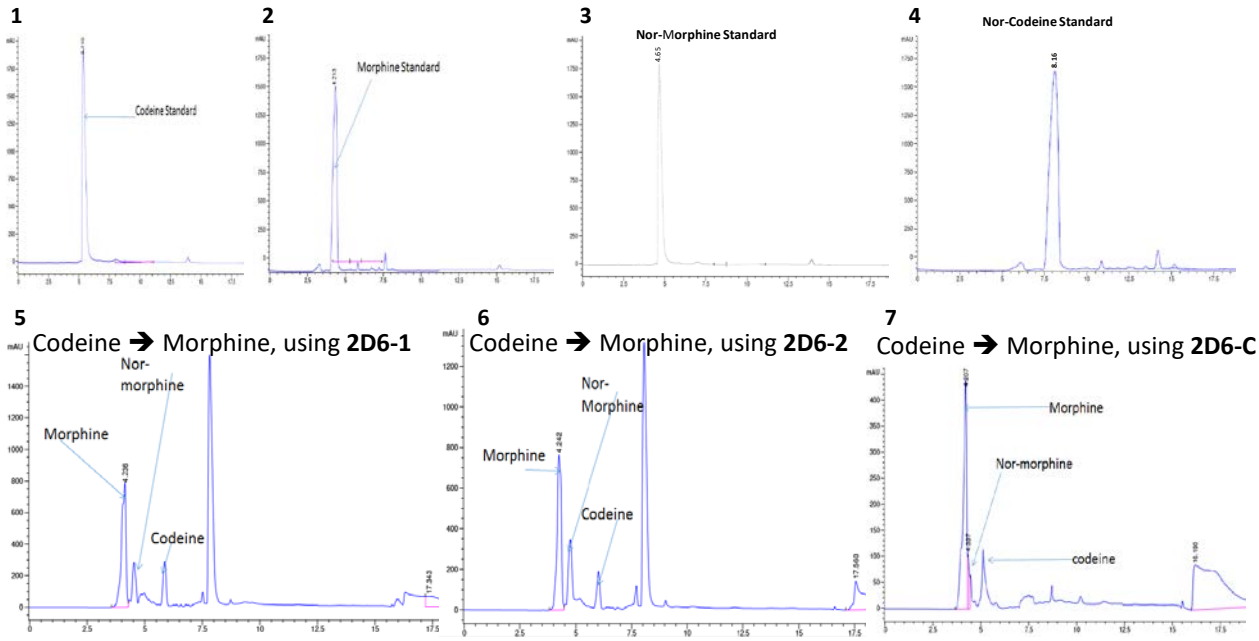


Figure 6.7. Panel 1: internal standard, codeine, with retention time 5.1 min. Panel 2: internal standard, morphine, with retention time 4.2 min. Panel 3: internal standard,nor- morphine, with retention time 4.65 min. Panel 4: internal standard, nor-codeine, with retention time 8.16 min Panel 5: codeine ➔ biotransformation, using yeast cells expressing 2D6-1, with retention time of morphine as 4.2 min. Panel 6: codeine ➔ biotransformation, using yeast cells expressing 2D6-2, retention time of morphine being 4.2 min. Panel 7: codeine ➔ biotransformation, using yeast cells expressing 2D6-C, retention time of morphine as 4.2 min. 10 µmoles of codeine, dissolved in water, was incubated with whole yeast cells containing the CYP2D6 variants for 72 h. The concentration of samples injected were kept constant. In chromatogram 7, morphine is seen to be the major peak. In chromatograms 5 and 6, the major peak is not that of morphine.

The results in Figure 6.7 would imply that the variant 2D6-C predominantly forms morphine (chromatogram 5) when compared with the wild type (2D6-2; chromatogram 5) and 2D6-1, the Val^{374} variant (chromatogram 3). The Val^{374} variant of CYP2D6 enzyme is sold as Supersomes by Corning-Gentest. Supersomes are used worldwide in the process of drug discovery by major pharmaceutical companies. The results from the chromatograms have been tabulated (see Table 6.1).

Table 6.1. Shows the percentage of morphine formed from the metabolism of codeine by the variants of CYP2D6 expressed in baker's yeast, *S. cerevisiae*.

Substrates	CYP2D6-1 peak area (mAu*)	CYP2D6-2 peak area (mAu*)	CYP2D6-C peak area (mAu*)
Morphine	800	775	440
Codeine	300	350	110
Nor-codeine	1600	1325	35
Nor-morphine	290	200	120
Total	2990	2650	705
% Morphine in the total products	26.8	29.3	62.4

Attempts have been made to rationalize the results obtained in Table 6.1 with the help of colleagues at the Indian Institute of Integrative Medicine (IIIM). The results are shown in Figures 6.8 to 6.10. A manuscript is in preparation incorporating these results.

1. Wild type CYP2D6 (2D6-1) (4WNT)

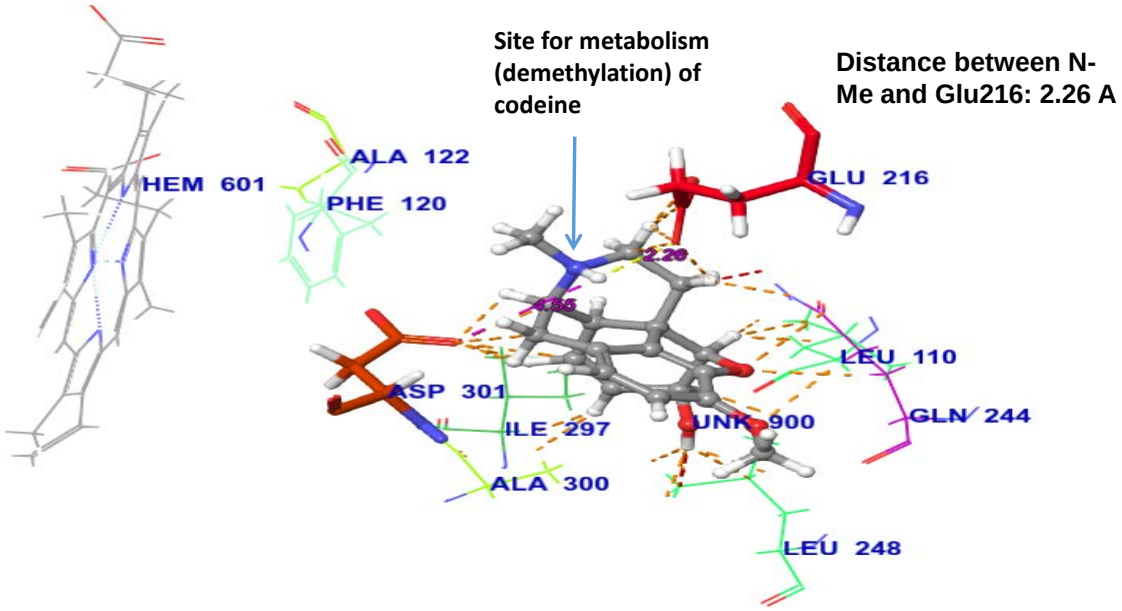


Figure 6.8. CYP2D6 (1) docking shows N-demethylation as a preferred site of metabolism. The interaction justifies the formation of norcodeine when 10µM of codeine was added to yeast cells bearing CYP2D6 (1) gene (4WNT). The distance between N-Me and Glu216:2.26A.

2. Mutated CYP2D6-2 variant (4WNT) – Mutations – V374M

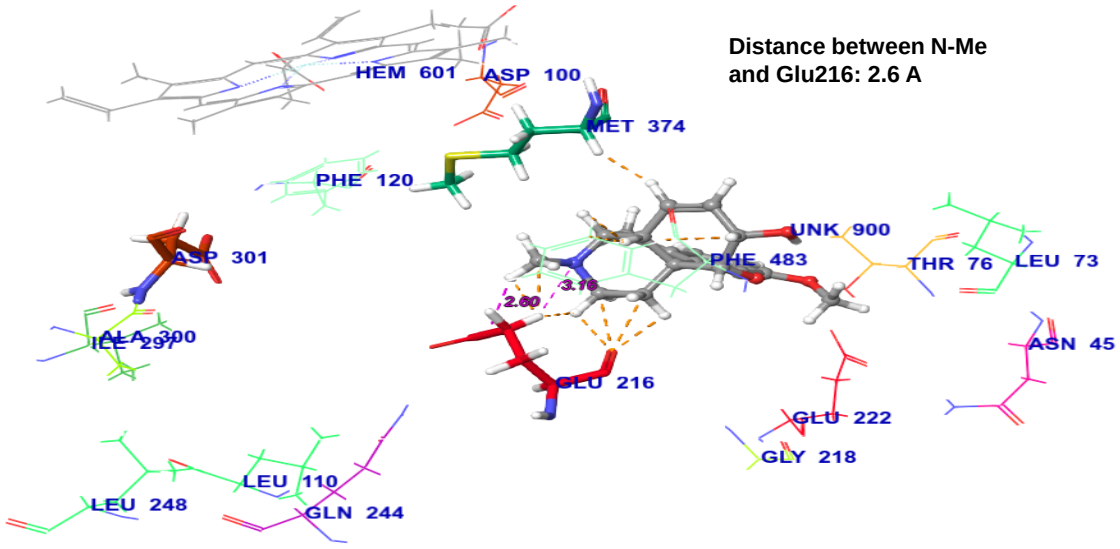


Figure 6.9. CYP2D6 (2) docking shows N-demethylation as a preferred site of metabolism. The interaction justifies the formation of norcodeine when 10µM of codeine was added to yeast cells bearing CYP2D6 (2) gene (V374). The distance between N-Me and Glu216:2.26A.

3. Mutated CYP2D6-C variant (4WNT) – Mutations – P34S/A122V/S486T

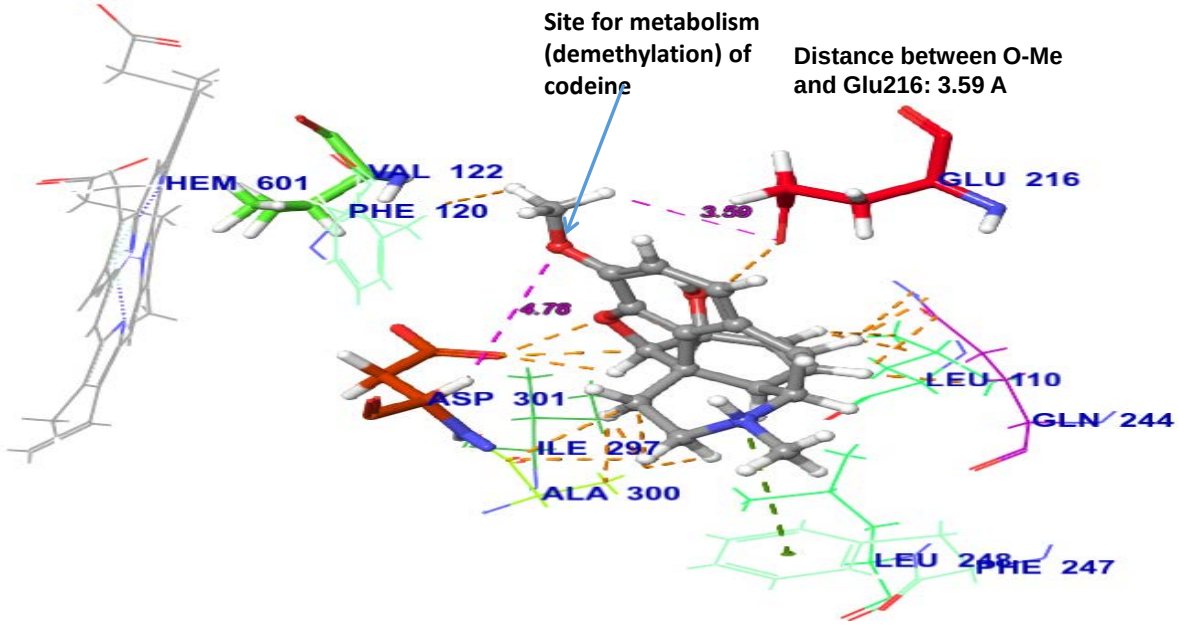


Figure 6.10. CYP2D6 (C) docking shows O-demethylation as a preferred site of metabolism. The interaction justifies the formation of norcodeine when 10µM of codeine was added to yeast cells bearing CYP2D6 (C) gene (PS4S/A122/S486T). The distance between O-Me and Glu216:3.59A.

Our efforts using yeast whole cells have resulted in the development of a reproducible preparative-scale biotransformation process for the conversion of codeine to morphine. According to the literature (Wang et al., 2015), Glu216 and Asp301 are important residues which are involved in de-methylation reactions. During docking, the interactions with these residues were closely observed. In the demethylation of codeine, Glu216 seems to play a crucial role.

Docking results have shown that in case of 2D6-1 (wild type) and 2D6-2 (V374M variant), the N-methyl group orients towards Glu216 residue, whereas OMe group is orienting away from this residue. However, in the case of 2D6-C (P34S/A122V/S486T) variant, the orientation of codeine was precisely reversed in the binding cavity of this enzyme resulting in the orientation of OMe towards Glu216, and N-Me away from this

residue. These observations validate our experimental results very well. There is nothing in the literature which would indicate that anyone has shown this type of studies with CYP2D6.

Figure 6.11 shows the TLCs of codeine to morphine biotransformation using the three CYP2D6 variant enzymes being expressed with live yeast cells.

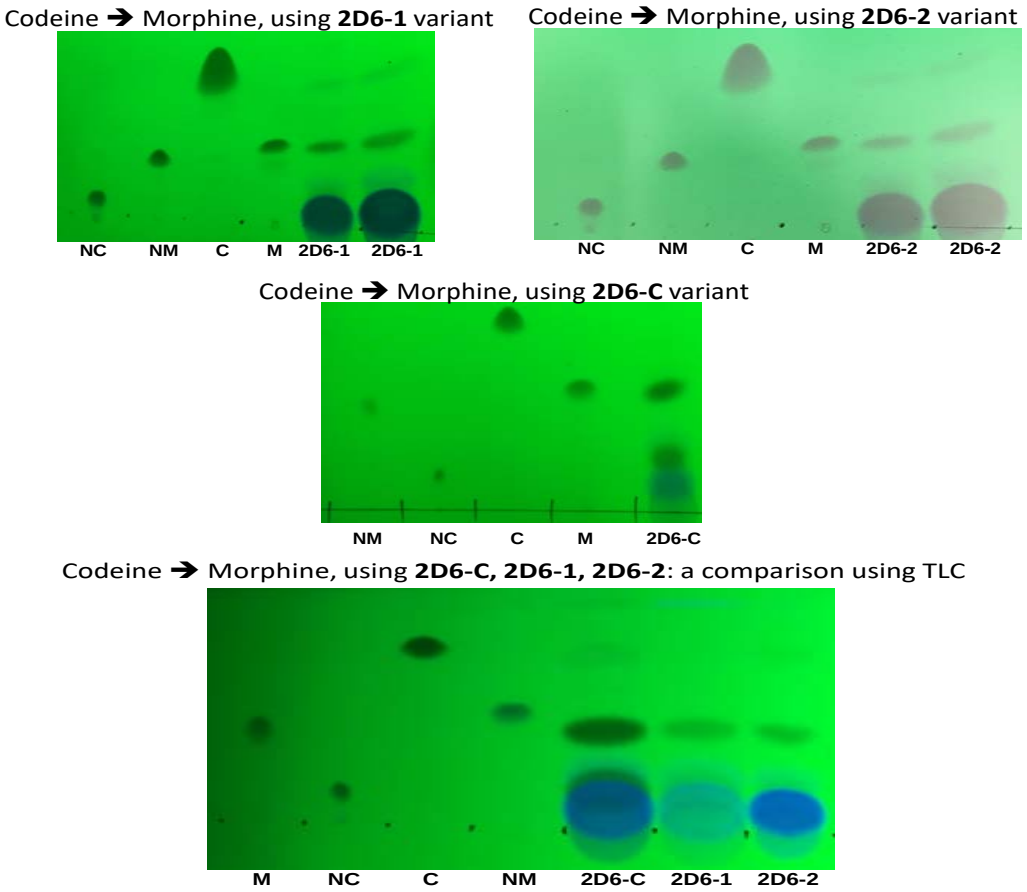


Figure 6.11. The TLCs provide qualitative pictures of the codeine to morphine biotransformation, using the yeast strains that express the three CYP2D6 variant enzymes, 2D6-1, 2D6-2 and 2D6-C. TLCs also show reproducibility of product formation. In all experiments, 10 µM (final concentration) of codeine was added to each yeast cell culture, bearing different CYP2D6 variants. After cell culture, the media were extracted with solvent; equal volumes (5 µl) were spotted on to lanes of the TLC plates [Sigma Aldri**ch (Z193275-1)]. Solvent system used for extraction, CHCl3:MeOH:NH3 = 36:1:0.6. C = codeine; M = morphine; NC = norcodeine; NM = normorphine.**

6.4.5 Discussion and future work

Three variants of CYP2D6 enzyme have been studied for their ability to convert codeine to morphine. From the results obtained, the CYP2D6-C variant could be classified as a rapid metaboliser of codeine. It was observed that more than 60% of codeine was converted to morphine. This is quite high compared to 27% and 29% conversion seen with 2D6-1 and 2D6-2 variants (Table 6.1). It has been reported in the literature that up to 15% conversion of codeine to morphine is normally observed within the human body (Berg-Perdersen et al., 2013). It was seen from the TLCs that with the wild type (2D6-1) and the Val^{374} variant (2D6-2) > 60% of the product was nor-codeine, codeine undergoing *N*-de-methylation to form nor-codeine (Berg-Perdersen et al., 2014). A similar trend is seen on the HPLCs where the peak for nor-codeine was the highest (Figure 6.7, Panels 3, 4, retention time of nor-codeine, 8.2 min).

We believe that the analysis of codeine to morphine biotransformation could be studied in such detail because of the stability of CYP2D6 expression in yeast strains which was afforded by the platform technology that has been created during work conducted for this thesis that allows stable expression of *CYP* genes from chromosomal loci of the yeast genome.

6.5 Metabolism of compound AZD-2014 using whole yeast cells that express human CYP3A4 (preliminary results)

6.5.1 Introduction

Cytochrome P450 (CYP) enzymes oxidise endogenous compounds, pharmaceutical drugs and other xenobiotics (Guengerich, 2007). CYP enzymes can exhibit both induction and inhibition in different reactions with drugs to enable their elimination after use. As discussed before, this often leads to drug-drug interactions (Heaths and Winkers, 2005). Dangers caused by drug-drug interactions are very common (Seymour, 1998). In order to analyse such interactions before seeking approval of a prospective drug from drug regulatory authorities,

(1) Firstly, one must determine which CYP enzyme is mediating the metabolism of a prospective drug, and

(2) Secondly, one must determine how much of a metabolite is being formed and then identify the metabolite(s) formed.

The reasoning behind this is

(a) The CYP enzyme that is mediating metabolism may be strongly inhibited by another approved drug so that one needs to be extra cautious about administering such drugs together, and

(b) A CYP enzyme mediated metabolite that is formed from a prospective drug is toxic for the human body.

Hence, drug regulatory authorities demand identification of a metabolite and the CYP enzyme responsible for metabolism.

6.5.2 Aim of experiments

We embarked on experiments to find if CYP3A4, which is the metabolizer of most approved drugs, also metabolizes a drug which is currently in clinical trials. The drug which was chosen was AstraZeneca's AZD-2014, an mTORC inhibitor. The main aim of these experiments was to see whether there could be an improvement in metabolite formation of AZD-2014 using whole yeast cells that express human CYP3A4. AztraZeneca had reported that they had obtained less than 10% conversion of AZD-2014 to metabolite(s) using the processes (i.e. recombinant CYP3A4 enzyme and also different types of recombinant whole cells expressing CYP3A4) that they had used.

6.5.3 Background to compound AZD-2014

AZD-2014 is a second generation dual inhibitor of mTORC-1 and 2. It is presently in the phase II/III clinical trials. This compound is reported to have shown high inhibition against mTORC-1 and 2. mTOR is an atypical serine/threonine kinase that is present as two complexes, complex 1 (C1) and complex 2 (C2). The first complex (mTORC 1) includes the proteins mTOR, Raptor, GβL and DEPTOR (DEP domain-containing mTOR-interacting protein) and it is inhibited by rapamycin. The complex is a master growth regulator that regulates and senses integrating different nutritional, cellular stresses and other metabolic factors. The second complex, mTORC-2, is composed of

mTOR, Rictor, GβL, Sin1, PRR5/Protor-1, and DEPTOR. Complex 2 controls cellular survival by activating Akt, a Ser/Thr kinase (Eyre *et al.*, 2014). Misregulation of mTOR is involved in many diseases including cancer, diabetes, and cardiovascular disease (Dowling *et al.*, 2010). Figure 6.9 shows the different biochemical pathways that relate to mTORC-1 and 2. It has been published that AZD-2014 inhibits the AKT/ mTOR signal transduction pathway without any negative-feedback on mTORC-2 in any type of tumour cells (Rastogi *et al.*, 2013).

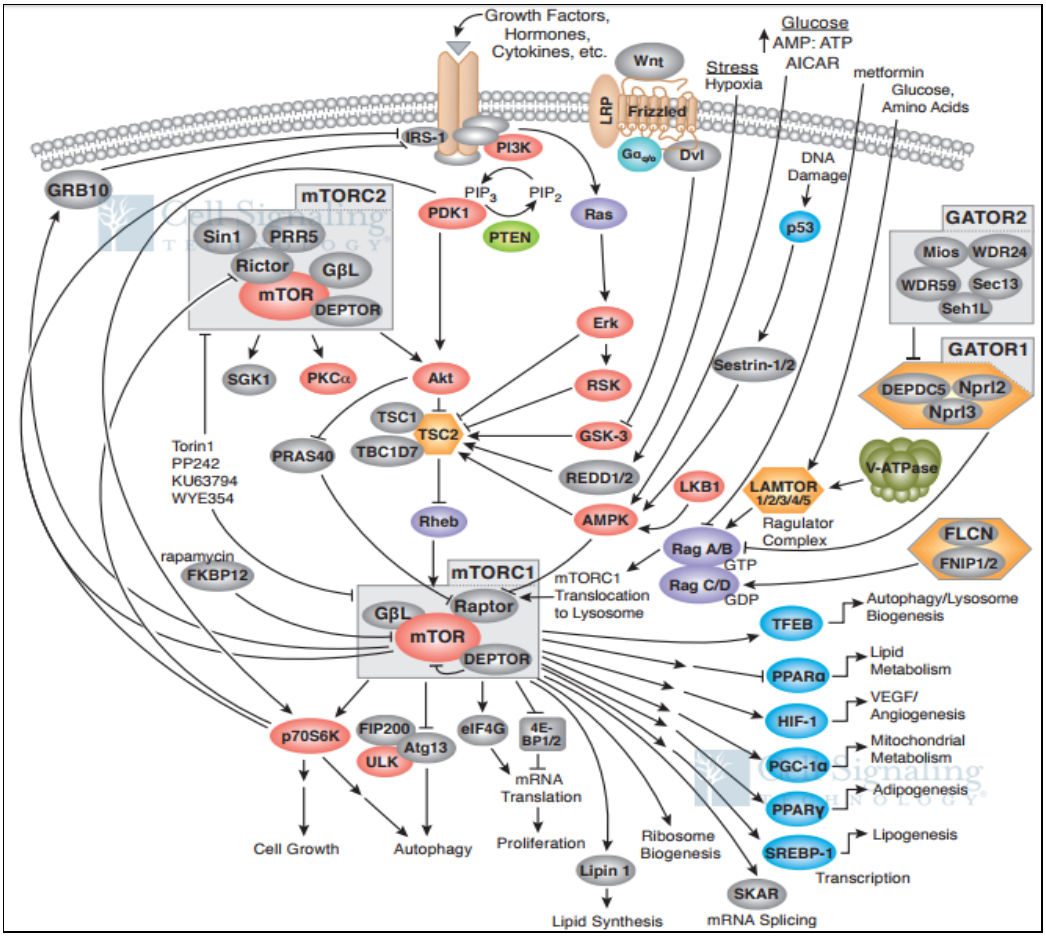


Figure 6.12. Biochemical pathways linked to mTORC-1 and 2 (Eyre et al., 2014).

It has been reported that CYP3A4 inhibition should be avoided during the use of mTORC inhibitors because CYP3A4 is the major CYP enzyme involved in the metabolism of this

class of compounds (Dancey et al., 2010; Laplante et al., 2012). We set out to find out if whole yeast cells expressing CYP3A4 could metabolize AZD-2014 and to what extent.

6.5.4 Results

The experimental procedure for this study was similar to the one described earlier in Section 6.3 of this Chapter. 10 µM (final concentration) of AZD-2014 (structure shown in Figure 6.13) was incubated in baffled shake flasks with whole yeast cells expressing CYP3A4 for different time periods.

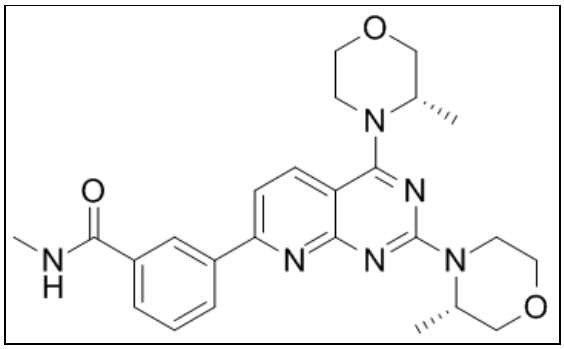


Figure 6.13. Chemical structure of AZ-2014.

The cultures showed accumulation of white crystallised particles at the bottom of the flask (Figure 6.14).

Figure 6.14. Shows accumulation of fine white crystalline particles at the bottom of the flask when AZD-2014 was incubated with CYP3A4-expressing yeast cells.

The picture in Figure 6.14 would indicate metabolite formation since the starting material, AZD-2014, is amorphous.

The results obtained from HPLC analysis of product formation are shown in Figure 6.15.

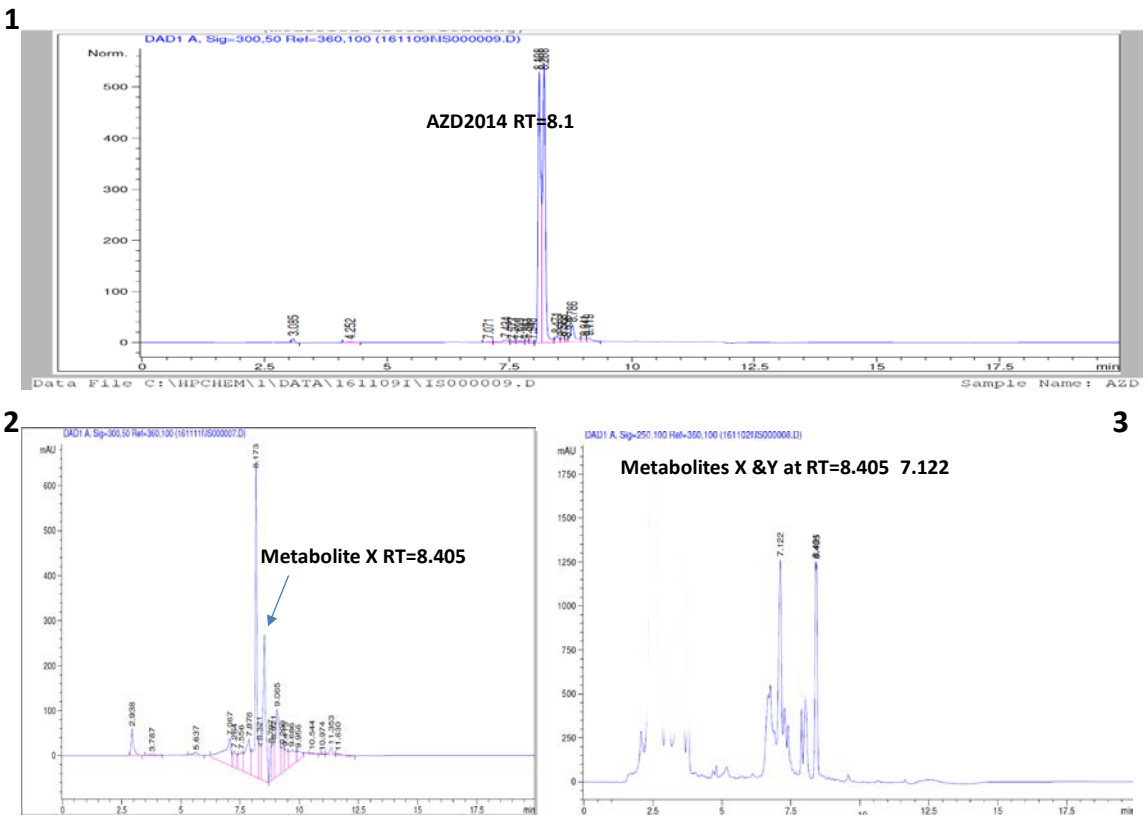


Figure 6.15. The chromatograms show the results from HPLC after compound AZD-2014 (supplied by AstraZeneca) was incubated with yeast cells expressing CYP3A4 in a YPD culture. The incubation time lasted from 48 h to 96 h. Chromatogram 1, retention time (RT) of AZD-2014 is ~8.1 min. Chromatogram 2, shows that besides AZD-2014, there was a product formed at 48 h which had a RT of 8.405 min. Chromatogram 3, after 96 h of incubation, two products could be seen, one with RTof 7.122 and the other RT of 8.405, and the AZD-2014 peak was greatly diminished.

The identity of the true metabolite of AZD-2014 has not been revealed in this report because of the confidentiality agreement with AstraZeneca over the compound.

However, the main interest of this study was to establish the percentage conversion of the AZD-2014 using whole yeast cells expressing the human CYP3A4 enzyme. Hence, TLCs were performed (Figure 6.16) to corroborate the HPLC results.

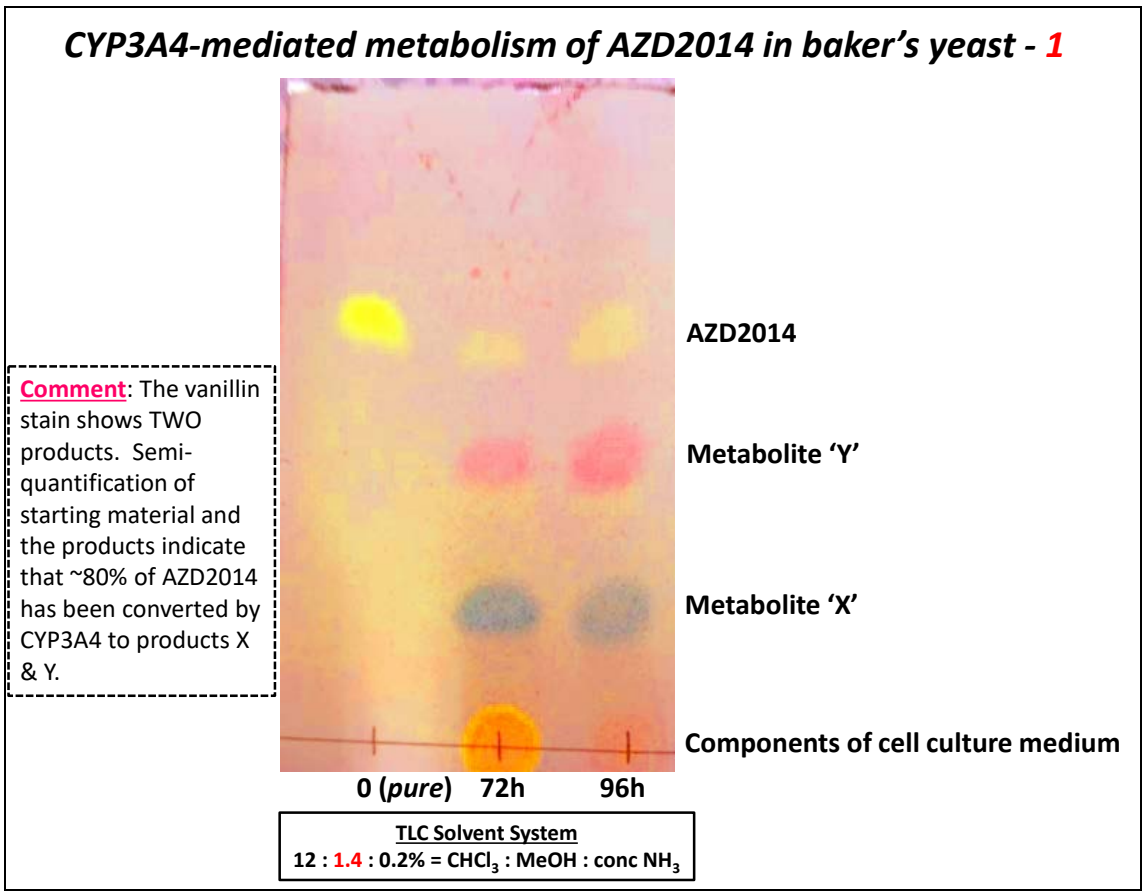


Figure 6.16 (1). The first TLC that shows the formation of metabolites from AZD-2014 after incubation with CYP3A4-expressing yeast cells over a time period of 96 h.

The results in Figure 6.16 (1) would indicate that more that 80% of AZD-2014 was converted to metabolites. This was confirmed by densitometric quantification (results not shown).

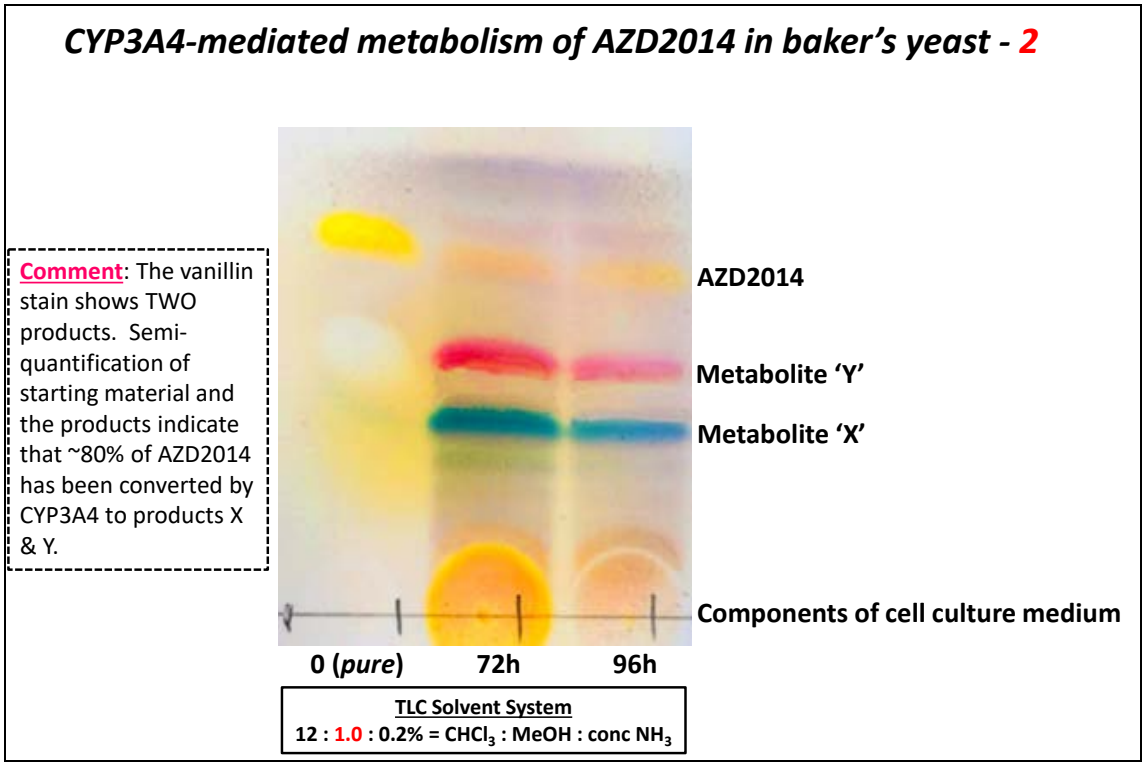


Figure 6.16 (2). The second TLC that shows the formation of metabolites from AZD-2014 after incubation with CYP3A4-expressing yeast cells over a time period of 96 h.

The results in Figure 6.16 (2) would again indicate that more that 80% of AZD-2014 was converted to metabolites. This was also confirmed by densitometric quantification (results not shown).

6.5.5 Future work

The results obtained clearly show that large amounts of metabolites could be quickly obtained from a potential medicine that is in development. Without the constraints set by AstraZeneca, one could have easily identified the metabolites.

6.6 Conclusions

In conclusion, herein I have demonstrated the ability of whole yeast cells overexpressing CYP1A1, CYP2D6 variants, and CYP3A4 enzymes to metabolize (i.e. bio-transform) different substrates. The high yields obtained in all biotransformation reactions, performed until now, have never been reported before.

(1) CYP1A1 was able to catalyse the biotransformation of the natural flavonoid chrysin to another natural flavonoid baicalein with more than 92% conversion.

(2) A CYP2D6 variant converted 62% of codeine to morphine; such high conversion to morphine has never been reported before.

(3) CYP3A4 mediated the conversion of more than 80% of AZD-2014 into two unknown metabolites; both metabolites play an important part in the clinical trials that are currently running.

These unique examples demonstrated in this study indicate the future opportunities for exploring the utility of these stabilized yeast cell based CYP enzymes for industrial production of medicinally important compounds and metabolite formation of medicines in development.

In summary, the stable CYP-expressing yeast strains ought to open up new exciting avenues in the area of (a) biotransformation and (b) drug metabolism, both being crucially important for the discovery of new and novel medicines.

Chapter 7 Establishment of a yeast system that stably co-expresses two or three different human CYP enzymes,– applications in CYP inhibition studies, drug-drug interactions and drug metabolism

7.1 Introduction

7.1.1 Background

Most high-value chemicals are plant products. These chemicals are formed usually from low value natural products widely occurring in plants. The transformation from a low value to high value chemical is often mediated by cytochrome P450 (CYP) enzymes. This process of conversion is referred to as the process of secondary metabolite formation. The metabolites are of high value mostly because they have unique medicinal properties but, unfortunately, are produced in nature in very low amounts. Usually, such metabolites are not amenable to convenient and affordable synthetic approaches since their formation always involves regio- and stereo-specific organic chemical reactions which comprise of multiple steps. The beauty of nature is that similar reactions are catalysed by CYP enzymes with the transformation occurring in a single step (Zhang et al., 2014).

Earlier in this study, the yeast *S. cerevisiae* was shown to be an organism which could be used to create powerful recombinant cell systems. In this thesis yeast has specifically been utilized as a heterologous expression system for the production of human CYP enzymes at high levels with enhanced activity. It was thought at the outset of this project that the similar intracellular features of baker's yeast to that of human cells would be a major

advantage that would facilitate efficient heterologous expression in yeast of human CYP enzymes with active site geometries identical to that present in human cells. Like human cells, yeast is a eukaryotic organism. Since it is unicellular, it is amenable to easier genetic modifications than human cells which are multi-cellular. It was conjectured that after introduction of human *CYP* genes into yeast, the human enzymes produced in yeast would behave just like the enzymes present in human cells. They would also be expected to be bound to the yeast endoplasmic reticular (ER) membranes which are structurally similar to the human ER membranes (Zimmermann et al., 2011).

However, it was also realized that there was a major drawback facing the production of these heterologous CYP proteins in yeast. It was the instability of the genetic information, which is foreign to yeast, being carried within the cells on extra-chromosomal plasmids that could prevent proficient protein synthesis and could lead to inconsistent production of human CYP enzymes. Although the genome of *S. cerevisiae* has completely been sequenced (Goffeau et al., 1996), yet until now there is still no complete understanding on how to optimise the production of foreign (i.e. heterologous) proteins/enzymes in yeast.

Yeast has been thought of as a system where not only foreign proteins could be made in bulk but also where important naturally occurring chemicals or their precursors could be made in large amounts (Sordon et al., 2016). Therefore, based on the knowledge of the yeast genome, there have been concerted attempts to develop biological tools, aimed at utilizing this organism, for construction of novel biosynthetic pathways within the yeast genome. The main focus of these attempts has been to establish methodologies that would

allow stable introduction of genes into the genome of yeast that would allow efficient production of proteins and chemicals (Mumberg et al., 1994, 1995).

The ability of baker's yeast to be transformed, with large DNA sequences, which can be stably maintained, has made it a powerhouse for sequencing the genome of any living organism. Baker's yeast has the capability of taking up efficiently multiple DNA fragments simultaneously. Homologous recombination allows this to take place in a single step (Larionov et al., 1996). Consequently, multiple fragments can be integrated into the yeast genome so that they could be propagated stably during cell division, without having to select for any extra-chromosomal plasmid in slow-growing selective medium.

7.1.2 Stability of genetic information, foreign to yeast, during propagation of yeast cells

Several techniques have been developed to open up the possibility of rapid assembly of genes, embodying large biosynthetic pathways, on the yeast genome. With the advancement of rapid gene synthesis, orchestrated by combinations of overlapping oligonucleotides, new biosynthetic pathways have been incorporated on to the yeast genome to synthesise novel chemicals (Benders et al., 2010).

An episomal 2-micron (2µ) plasmid should, by definition, provide multiple (at least >10 but possibly 40-60) copies of plasmids per cell allowing strong expression of plasmid encoded genes (Rizvi et al., 2017). During the course of the studies reported in this thesis, this assumption turned out not to be true. On comparison of expression of a *CYP* gene from an episomal plasmid contained in a yeast strain with strains that harboured integrated copies of the same gene, it was noticed that instead of expressing multiple copies of a

gene, an episomal plasmid expressed much less CYP enzyme than a single copy gene, integrated at a chromosomal locus. This would indicate that an episomal plasmid does not deliver stable CYP expression within cells. It has been suggested that the inherent instability of episomal 2µ plasmids can be burdensome for cells and could result in cellular toxicity and cell death (Parent et al., 1985).

Although low copy number plasmids, which are used to construct yeast artificial chromosomes (YACs) afford more stable expression relative to episomal 2µ plasmids, they suffer from low gene expression. Hence, low copy number plasmids have limited use. Gjuracic and Bruschi (2005) have reported that retention of YAC constructs within live yeast cells can be problematic because it is difficult to maintain the selection pressure on an extra-chromosomal entity, which is the YAC. YACs can be maintained only in selective synthetic defined (SD) minimal medium in which yeast cells do not grow as well as in non-selective complete YPD medium. In fact, cells in SD medium grow poorly.

One of the major achievements of the studies described in this report is the enhancement of expression levels of heterologous human *CYP* genes by using DNA constructs which permit chromosomal integration. It has been noticed by others that during the simultaneous expression of multiple genes that belong to a biosynthetic pathway of a natural product, balancing the expression levels of individual genes is quite important in order to achieve an optimal flux through a biochemical pathway and also to avoid the accumulation of undesired chemical intermediates or by-products that may be formed during biosynthesis (Pitera et al., 2007). The two main aspects which are considered before chromosomal integration of a gene are copy number of the gene that is introduced into the genome and the strength of the inducible or constitutive promoter.

The studies described in this report has used human genes, synthesised with yeast biased codons, to overcome the problem of epigenetics, that is, to avoid any sudden changes in chromosomal structure after introduction of a foreign gene into host cells that would ultimately lead to the lowering of gene expression. Manifestation of epigenetic effects after introduction of foreign genes into host organisms has been discovered in several systems, including that of *E. coli*, *Lactococcus lactis* (Thompson and Gasson, 2001), and *S. cerevisiae* (Yamane at al., 1998, Thompson and Gasson, 2001). Most researchers observed epigenetic effects that involve silencing of gene transcription (Ottaviani et al., 2008). Differential transcriptional silencing effects were observed at different chromosomal loci of these organisms (Fang et al., 2011).

7.1.3 'Reiteration recombination' method for chromosomal integration of genetic information into yeast

Recently, the 'reiterative recombination' method has combined high efficiency with nuclease induced homologous recombination, for integration of multiple gene expression cassettes either (a) randomly into different chromosomal loci or (b) targeted repetitively to a particular chromosomal locus. The genes, in the expression cassettes that have been reported, represent pathways to the syntheses of different chemical entities. Homologous recombination of genes, in the 'reiterative recombination' method, can occur stepwise with repetitive use of a replaceable selection marker. This occurs at a specific chromosomal locus. Alternatively, one could use the 22 bp delta (δ) elements of yeast retrotransposons (Tys) to integrate multiple genes in different chromosomal loci. There are around 50 Tys in yeast and hundreds of δ elements.

In one study, a two-gene biosynthetic pathway for production of the chemical 1,2-propanediol was constructed using δ integration cassettes. The δ elements that were chosen are part of the long terminal repeats (LTRs) of the Ty1 and Ty2 retrotransposons of the yeast *S. cerevisiae*. In the *S. cerevisiae* chromosomes there are dispersed several hundred δ elements which exist alone or are associated with Ty elements. A plasmid bearing a heterologous (foreign) gene and containing a δ element can, in theory, allow tens (possibly hundreds) of integrations at the different chromosomal loci, where a Ty element exists, via yeast transformation. Although transformants are selected in minimal medium, the resultant strains can be grown in full medium because the plasmids are integrated into the yeast genome, and once integrated there is no possibility of plasmid loss. In this particular case, strains with only three copies of each gene showed the highest level of production of 1,2-propanediol (Lee and Da Silva, 2006).

7.1.4 'Cocktail integration' method for chromosomal integration of genetic information into yeast

Cocktail integration is another method of chromosomal integration of genes in yeast which has been described by Yamada et al (2010). Their work was related to simultaneous integration of three genes, *BGL*, *EG* and *CBH*, involved in the production of cellulose. This again involved the method of delta (δ) integration. Simultaneous introduction of the first copy of the cocktail of three genes resulted in the production of higher levels of cellulose compared to the experiment when each gene was introduced individually using a δ element. Introduction of the second copy of the cocktail of genes, at the same time, produced higher cellulose levels than the first copy cocktail. However, after the third copy of the cocktail of genes was introduced, it was found that there was saturation in the levels

of gene expression, that is, there was the same cellulose level as had been seen in cells containing the second copy cocktail.

7.1.5 'Recyclable integration' method for chromosomal integration of genetic information into yeast

Codruta et al (2011) have studied the production of plant terpenoids in yeast. They were able to improve production of specific terpenes in yeast using 'recyclable' integration cassettes. It was found that there was around 60% augmentation in protein synthesis with appropriate choice of chromosomal loci.

As seen earlier in this report, the yeast *URA3* gene has been used as a selection marker for selection of plasmids (both episomal and integrative) during yeast transformation and for maintenance of only extra-chromosomal plasmids in minimal SD medium during propagation (i.e. growth) of yeast cells. The functional *URA3* gene can be used for repetitive 'recycling' (Alani et al., 1987) during consecutive gene integrations. For example, after the first integration with a *URA3* integrative plasmid, host cells containing a non-functional *ura3* gene become $URA3^{+}$. In order to convert the $URA3^{+}$ cells back to $ura3^{-}$, the cells are transformed with a truncated non-functional *ura3* (*Δura3*) gene and the transformant cells are then grown in the presence of the chemical 5-fluoroorotic acid (5-FOA) to select for the presence of only $ura3^{-}$ cells. The Ura3 protein, coded for by the functional *URA3* gene, converts 5-FOA to the toxic compound 5-fluoro-UMP. Thus, cells which contain a functional *URA3* gene die and only the cells containing a non-functional *ura3* survive and multiply in a uracil containing synthetic SD medium. Thus, the *URA3* gene can be used for repetitive counter-selection (i.e. 'recycled') in the presence of 5-

FOA, for introduction of a new gene or a new copy of the same gene using a series of *URA3* integration plasmids.

In order to repetitively 'recycle' virtually any desired selection marker (not only *URA3*), the bacteriophage-derived *Lox*P-*Cre* recombinase system has been developed (Hoess & Abremski, 1985; Sauer, 1987; Guldener et al., 2002). It exploits the site-specific activity of the *Cre* recombinase which efficiently removes markers by flanking them with a targeted *Lox*P sequence. However, the *Lox*P-*Cre* system has a major limitation. Namely, it requires the co-expression of a *recombinase* gene which is borne on a plasmid that must contain an additional selection marker (Schorsch et al., 2009). Moreover, the repetitive (i.e. 'recyclable') use of this system can cause major chromosomal rearrangements.

7.1.6 Comparison of expression from episomal plasmid and after chromosomal integration: a published study

Barun et al (2012) have co-expressed CYP2D6 and CYP3A4 with CPR from both episomal and integrative plasmids. The amounts of CYP proteins obtained were 4 to 10 fold more when the *CYP* and *CPR* genes were co-expressed from a multi-copy episomal plasmid than when they were co-expressed from a single integrated copy from the same chromosome. Moreover, the multi-copy plasmid transformants showed a 50 to 70-fold increase in CYP activity compared to the single copy integrated strains. This is in stark contrast to our findings. However, it should be pointed out that the amounts and activities of CYPs produced by Barun *et al* (2012) were negligible compared to what has been presented in this report.

7.1.7 Drug metabolism, a crucial factor in Drug Development

Metabolism of drugs mediated by CYP enzymes is an extremely important factor in determining the occurrence of drug-drug interactions. These determinations have been greatly aided by the availability of microsomal (ER membrane bound) enzymes that have been isolated from recombinant organisms, such as insect cells, and which are available commercially from Corning and Invitrogen (Thermo Fisher). The enzymes produced by Cypex from bacterial cells are bound to the bacterial cells' periplasmic membranes; prokaryotic bacterial cells do not contain ER membranes.

The CYP microsomal enzymes that are routinely used for drug-drug interaction studies are CYP3A4, CYP3A5, CYP2D6, CYP1A2, CYP2C8, CYP2C9 CYP2C19, CYP2A6, CYP2B6 and CYP2E1. The intrinsic and extrinsic factors that affect the activities of these CYP enzymes are described in Figure 7.1. The enzymes CYP1A1, CYP1B1, CYP2J2 and CYP4F3 have been used less often for drug-drug interaction studies in the past. However, they are likely to be used more widely if their availability increases from commercial sources.

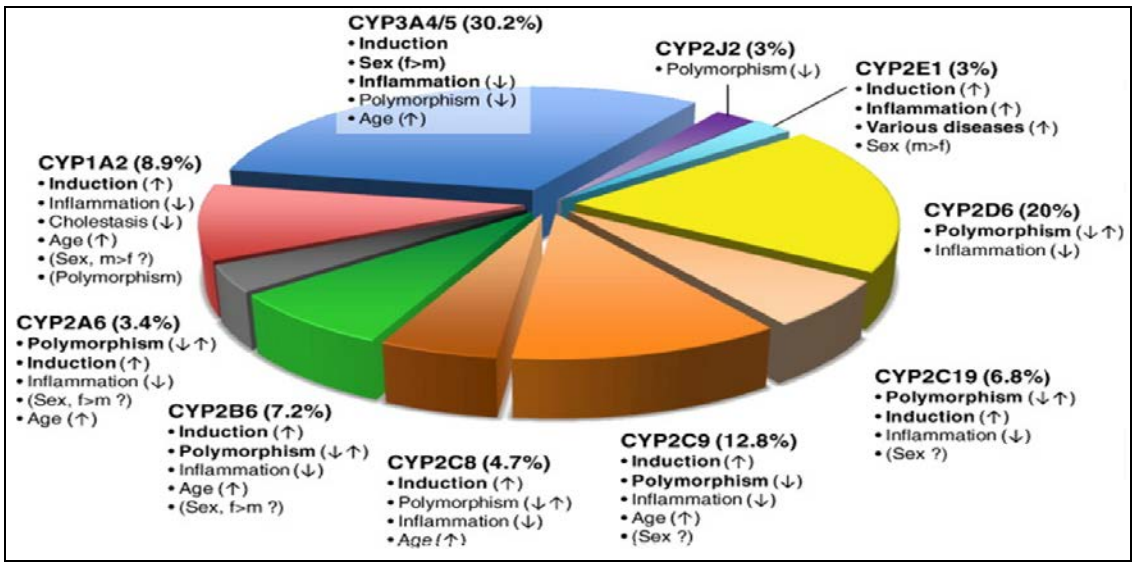


Figure 7.1 Pie-chart showing the CYP450 isoforms and factors influencing variation of their activities. Adapted from Pharmacology and Therapeutics, Ulrich et al., 2013.

7.2 Prologue to the studies in Chapter 7

7.2.1 Summary of findings until now

In Chapter 3, it was seen that the human *CYP* genes, chemically synthesized using yeast biased codons, gave enhanced expression of CYP enzymes compared to the native *CYP* genes that had been isolated from a human liver cDNA library.

In Chapter 4, the effects of integration of a single copy of the *CYP* gene, synthesized using yeast biased codons, at different chromosomal loci of yeast were clarified; it was seen that the *HIS3* locus produces more CYP enzyme than from the *URA3* or the *ADE2* loci.

In Chapter 5, it was shown that a second copy of a *CYP* gene further enhances the levels of CYP enzyme when compared with yeast strains that contain only a copy of the gene.

In Chapter 6, whole yeast cells expressing specific CYP enzymes, at high levels, were used for specific biotransformation reactions. The widely available, naturally occurring flavonoid chrysin was converted in >90% yields by human CYP1A1 to bicalein, which is another natural flavonoid but available only in miniscule amounts in nature. The wild type human CYP2D6 (Met^{374}) and the Val^{374} variant mediated only ~30% conversion of codeine to morphine whereas, surprisingly, the variant S^{34},V^{122},T^{486} was found to be a far superior metabolizer of codeine. Whole yeast cells expressing human CYP3A4 were used to find out if it was the CYP isozyme that was responsible for metabolite formation of AZD2014, an mTOR inhibitor, which is currently in Phase II/ III clinical trials.

7.2.2 Drug-drug interactions

There are many drugs that are crucial for therapeutics and treatment of diseases, which either inhibit or induce the activity of CYP enzymes. A CYP that is induced by drug (A) may also participate in the metabolism of drug (B). In the presence of drug (A), the dosage of the drug (B) must be enhanced to achieve a therapeutic effect within a certain time frame. In parallel, if a drug (C) were to repress the activity of a CYP at the transcriptional level and if the CYP were to be involved in the metabolism of drug (D), the dosage of drug (D) would have to be reduced. If the dosages of drugs (B) and (D) were not to be altered, severe side effects would occur (Lu et al., 2008). Hence, knowledge of this type of drug-drug interaction is crucial for the process of gaining regulatory approval (Prueksaritanont et al., 2013).

7.2.3 True inhibition of a CYP enzyme in the presence of other CYPs

In order to find the true inhibition of a CYP enzyme by a particular compound in vivo, there is another major issue which is often encountered. It can happen when a compound (A) is a verified substrate of CYP(1) enzyme, which implies that it is responsible for the metabolism of drug (A) allowing its clearance from the body. However, compound (A) also potently inhibits, in vitro, another CYP isozyme CYP(2). In this scenario, it is unlikely that the metabolite of drug (A), mediated by CYP(1) enzyme, is an inhibitor of CYP(2) enzyme and, hence, drug (A) as such cannot be acting as a potent inhibitor of CYP(2) in vivo. It is impossible to see such a phenomenon using isolated microsomal enzymes. Such problems have been exemplified through studies with the drug

metoprolol, a CYP2D6 substrate (Flockhart et al., 2002), and another drug warfarin which interacts with multiple CYP enzymes, CYP1A2, CYP2D6, and CYP3A4 (Weinshilboum et al., 2003). That's why the pharmaceutical industry relies more on human liver microsomes, which contain a conglomeration of CYPs, rather than on isolated microsomes from recombinant cells.

In Chapter 5, two copies of *CYP* genes were introduced into the genome of baker's yeast. The results had shown the creation of a potentially marketable asset. After having compared the activities of the yeast produced enzymes (Sacchrosomes) with the activities of the commercially available CYP enzymes (from insect and bacterial cells), which are already available in the market, it was clearly seen that Sacchrosomes are by far the best. On the basis of the studies described, we thought it would be possible to create novel systems that would allow the true evaluation of potential CYP inhibitors by constructing yeast strains that co-express 2 to 3 different human CYP enzymes, within the same cells from different chromosomal loci.

7.3 Outline of Chapter 7

In this Chapter (Chapter 7), based on the results obtained previously, integration of two or more different *CYP* genes in different chromosomal loci of baker's yeast were attempted to find the true CYP inhibitory potential of a compound in the presence of other CYPs. This was attempted as experiments using isolated microsomal CYP enzymes can lead to misleading data, as explained below. The *CYP* genes that were selected for these cellular studies were *CYP3A4*, *CYP2D6*, *CYP2C19* and *CYP1A2*. They are the main *CYPs* that are expressed in the liver.

The possibility of inhibition of CYP enzyme(s) by potential medicines is a very important aspect in drug development. The basic aim of this Chapter was to develop a yeast-based cellular system that would improve the status quo by facilitating CYP inhibition studies very early on in the development of new medicines. Because of their expense, HLMs are used at a much later stage in the pre-clinical/ clinical interface of drug development.

7.4 CYP3A4, CYP2D6, CYP2C19 and CYP1A2 enzymes

The CYP3A4 protein accounts for 28-40% of total hepatic CYP450 content. It is clinically important because it catalyses the metabolism of ~40% of all medicines that have been approved until now. The CYP3A5 isozyme, structurally very close to CYP3A4, is responsible for the metabolism of another 15% of drugs (Cheng et al., 2009).

CYP2D6 is expressed in many tissues including the liver, brain, breast, lungs kidney, and placenta. The expression of CYP2D6 is reported to be relatively low in the liver compared to the levels of CYP3A4. Although it constitutes 2-5% of total hepatic CYP proteins, it is involved in the metabolism of ~25% of therapeutically useful drugs such as codeine, bufuralol, debrisoquine, propafenone, morphine and others. One important feature of CYP2D6 is the presence of at least one basic nitrogen atom at a distance of 5-7 A° (angstrom) from the site that allows oxidation of substrates (Beijnen et al, 2013).

CYP2C19 is a member of the CYP2C subfamily which accounts for over 20-25 % of the total CYP proteins in the human liver. CYP2C family members are involved in the metabolism of ~20% of the approved drugs in the market. CYP2C19 substrates are neutral or very weakly basic with two or three H-bond donors or acceptors at 4-5A° and 5-8A°

from the site of metabolism. It metabolises omeprazole, imipramine diazepam and other drugs (Beijnen, 2013).

The CYP1A2 enzyme represents ~10% of the total CYP content in the human liver. It has only one H-bond donor site and therefore there are fewer drugs which are substrates of CYP1A2. It plays a part in the metabolism of ~4% of the drugs in the market. They include tacrine, caffeine, tamoxifene, propafenone and other related drugs. Besides being involved in the metabolism of important drugs, CYP1A2 also has a role in the metabolic activation of polyaromatic hydrocarbons (PAHs), aromatic and heterocyclic amines (Beijnen, 2014).

7.5 Yeast strains constructed for use in Chapter 7

In Chapter 4, plasmids containing a *CYP* gene were integrated at different chromosomal loci, *ADE2*, *HIS3* and *URA3*, of a defined yeast strain YY7 or YAB79. The resultant yeast strains, containing one copy of a *CYP* gene, were used to compare CYP enzyme activities that these strains expressed with strains that harboured an episomal plasmid.

In this Chapter (Chapter 7), the same *CYP* gene bearing integrative plasmids were used for further integrations that resulted in the incorporation of multiple *CYP* gene expression cassettes on different chromosomal locations. As stated in Chapter 4, the expression of all human *CYP* genes, including that of *CYP1A2*, *CYP3A4*, *CYP2D6* and *CYP2C19* are driven by *ADH2* promoter. The yeast integrated plasmids that were used to create the new yeast strains in this Chapter are listed in Table 7.1.

Table 7.1. The integrative plasmids that were used for the construction of single-copy *CYP* gene bearing yeast strains that would allow further

introduction of a second and third copy of different CYP genes into yeast strains.

Locus	Plasmids encoding *CYP1A2_yc* gene	Plasmids encoding *CYP2C19_yc* gene	Plasmids encoding *CYP2D6_yc* gene	Plasmids encoding *CYP3A4_yc* gene
ADE2				YIpAdeADH2S/CYP3A4
HIS3	YIpHisADH2S/CYP1A2	YIpHisADH2S/CYP2C19	YIpHisADH2S/CYP2D6	
URA3		YIpUraADH2S/CYP2C19	YIpUraADH2S/CYP2D6	YIpUraADH2S/CYP3A4

The yeast strains, that contained a single copy of a *CYP* gene and which were used as starting points for further integrations, were:

(i) YAB79::1A2(HISS3),

(ii) YAB79::2D6(HISS3),

(iii) YAB79::2C19(HISS3), and

(iv) YAB79::3A4(ADE2).

The availability of five distinct auxotrophic markers in the strains YY7 and YAB79 was taken advantage of for the introduction of further copies of different *CYP* genes. In YY7 cells, only one of the auxotrophic markers (*LEU2*) was occupied by the *ΔhRDM* gene which would easily allow four different *CYP* genes to be introduced into the yeast strain. In the YAB strain, two of the auxotrophic markers (*LEU2* and *TRP1*) had been used up by the *ΔhRDM* and cytochrome *b5* genes so that three different *CYP* genes could be introduced. Besides this, different antibiotic resistance genes, which cause resistance of

yeast cells to potent antibiotics that kill yeast cells, could be used for incorporating more *CYP* genes into the yeast chromosome.

Yeast strains that were created after integration of a second and a third *CYP* gene, for this study, were:

(d) YAB79::1A2(HIS3), 2C19(URA3)

(e) YAB79::1A2(HIS3), 3A4(URA3)

(f) YAB79::1A2(HIS3), 2D6(URA3)

(g) YAB79::2D6(HIS3), 3A4(URA3)

(h) YAB79::2C19(HIS3), 2D6(URA3),

(i) YAB79::2C19(HIS3), 3A4(URA3),

(j) YAB79::3A4(ADE2), 2C19(HIS3), 2D6(URA3),

(k) YAB79::3A4(ADE2), 1A2(HIS3), 2D6(URA3)

The yeast transformation technique used in these experiments was a high-efficiency yeast transformation protocol (Amberg et al., 2006). The yeast transformants, obtained from an integration of each new *CYP* gene bearing plasmid, were screened (i.e. selected) for the best CYP enzyme producers using fluorogenic substrates that act as substrates for the specific CYPs that were used. The best expressing yeast clones were streaked out on SD minimum medium agar plates, with the required selection markers. The plates were incubated for 3 days at 30°C to ensure that the plasmid had been retained, after integration, inside the yeast genome. Scoopful of cells, which grew on SD plates, was taken for inoculation in full medium YDP broth (i.e. liquid culture). YPD is a complex rich medium that is used for routine yeast growth. It contains 1% bacto-yeast extract, 2%

bacto-peptone, 2% glucose, as defined in Chapter 2. YPD medium cannot select for the presence of extra-chromosomal plasmids within cells.

5 ml of YPD broth was transferred into wells of 6-well sterile plastic plates. Then, an inoculum (i.e. scoopful of cells) from freshly grown agar plates, containing different yeast strains, was added to the different wells of the 6-well plate containing 5 ml of YPD broth. 2% adenine was added to supplement the small amount of adenine that is present in YPD but which is rapidly used up by the cells' biomass.

The 6-well plates were transferred to a shaking incubator, set at 30°C, for overnight growth of cells. After 12 h, glucose is used up by yeast cells and full induction of the *ADH2* promoter occurs. This allows expression of the CYP enzyme. The optical density at 600 nm (i.e. OD_{600}) was measured from each of the incubated cultures after 18 h of (overnight) growth. Cells usually attained an OD_{600}/ml of around 25. The cells were transferred into 2 ml Eppendorf tubes and were harvested. The cell pellets were washed three times with 500 µl TE buffer (50 mM Tris-HCl, 1 mM EDTA, pH 7.4) by spinning down the cells at 13,000 rpm, each time for 1 min. Cells were finally re-suspended in 450 µl of TE buffer to measure the kinetics of a CYP enzyme reaction with its substrate.

7.6 Assay to determine the activities of CYP enzymes

50 µl of cell suspensions were transferred into a sterile 96-well microtiter plate to which 50 µl of a substrate mixture (depending on the substrate for a specific CYP) was added. The parameters for the fluorescence plate reader (Synergy HT BioTek) were set using the appropriate extinction/emission filters and appropriate gain sensitivity settings to obtain

the best kinetic output from the plate reader. The plate was incubated at 30°C for 30 sec before the fluorescence emissions were measured.

7.7 IC_{50} determinations for inhibition of CYPs, using fluorogenic substrates

The protocol for IC_{50} determinations of compounds inhibiting CYPs has been described in Chapter 2 (Materials and Methods). A fluorogenic substrate was used to determine the potency with which a test compound inhibits a recombinant human cytochrome P450 (CYP) enzyme.

Yeast cells, expressing a CYP enzyme, were incubated in triplicate with a known CYP inhibitor, for example, quinidine (a 2D6 inhibitor), furafylline (a 1A2 inhibitor), ketoconazole (a 3A4 inhibitor) and ticlopidine (a 2C19 inhibitor) for 30 min. After incubation, the kinetic activity of the CYP enzyme was measured in the presence of a fluorogenic substrate. All data represented shows n= 3 ± SEM.

Individual CYP assays were performed using a 96-well microtiter plate. All assays included a negative control (i.e. no compound) and eight concentrations of test compound in 3-fold serial dilutions; 30 µM, 10 µM, 3 µM, 1 µM, 0.3 µM, 0.1 µM, 0.03 µM, 0.01 µM. The fluorescent metabolites were measured and the kinetics of metabolic reactions is represented graphically in Figures that follow. The bar charts represent comparison of fluorescence values obtained at a certain fixed time point of the CYP-mediated reaction on its substrate.

The results may allow us to answer the following questions:

(a) Are the enzyme activities of individual CYPs the same when two enzymes are expressed together as when they are individually expressed or is there a 'crowding effect' when two or three enzymes are co-expressed?

(b) Can co-expression of two CYPs affect the ability of a compound to inhibit one or the other?

(c) Can cells which co-express two CYPs be useful in screening a library of compounds for potential CYP inhibition?

7.8 Activities of individual CYPs when co-expressed in yeast (a) 2C19 & 1A2, (b) 3A4 & 1A2, (c) 2D6 & 1A2, (d) 2D6 & 2C19 compared with activities of 1A2, 2C19, 2D6, 3A4 which are expressed individually from a single integrated copy

For these studies, two separate *CYP* genes were integrated at the *HIS3* and *URA3* chromosomal loci of the yeast strain YAB79 which contains not only a modified gene of the human P450 reductase (ΔhRDM) but also the gene for cytochrome b5.

CYP1A2 was co-expressed with another CYP isozyme,

(a) CYP3A4,

(b) CYP2C19 and

(c) CYP2D6,

in the yeast strain YAB79.

On the other hand, CYP2D6 was co-expressed with CYP2C19, once again in the strain YAB79. This was with the view of confirming that co-expression of cytochrome b5 does

not have a deleterious effect on the expression of CYP2D6 enzyme as has been reported previously (Henderson et al., 2015).

The enzyme activities of CYP1A2, CYP3A4, CYP2C19 and CYP2D6 were measured, using fluorogenic substrates, when they were co-expressed with another enzyme from another chromosomal locus, and were compared with the activities of the same enzymes when they were expressed individually from the *HIS3* chromosomal locus.

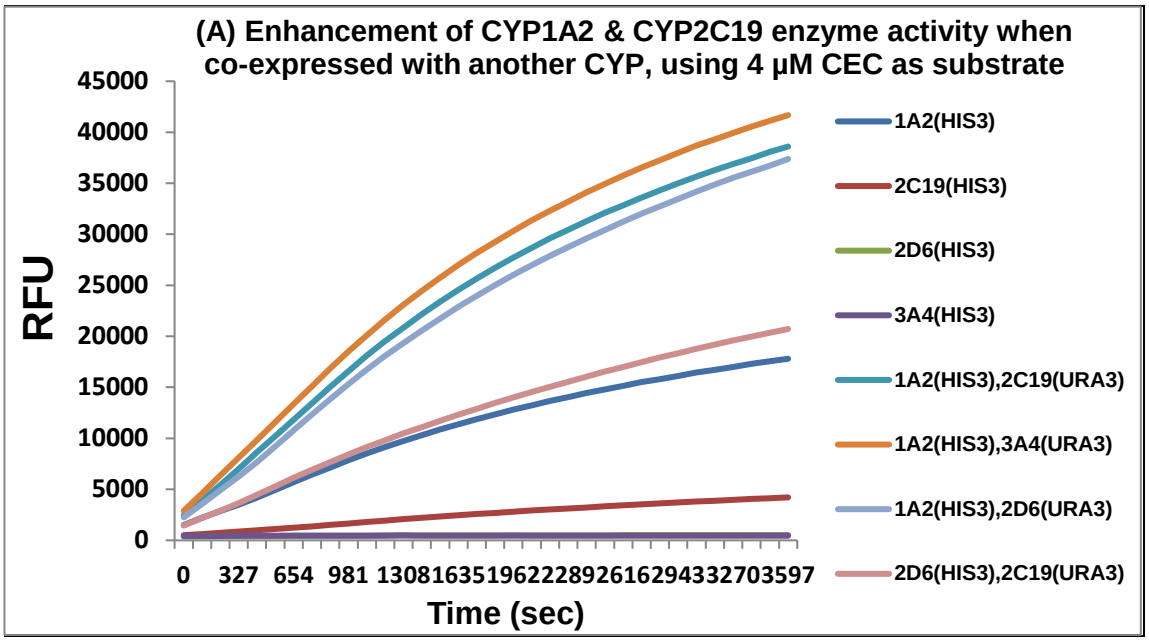


Figure 7.2. The graphs show the activities of CYP1A2 andCYP2C19 enzymes when co-expressed with a second CYP enzyme, in the strain YAB79. CEC is a fluorogenic substrate that primarily measures CYP1A2 and CYP2C19 activities. The activities are compared with the strains that express single copy *CYP* genes. The graphs represent the average of results obtained from three independent experiments.

Figure 7.2 shows that the activities of CYP2D6 and CYP3A4 are not measureable, using the fluorogenic substrate CEC. However, the activities of CYP1A2 and CYP2C19 are quantifiable. It appears that the CYP2C19 activity is greatly enhanced when co-expressed with CYP2D6. In parallel, CYP1A2 activity is remarkably improved when it is co-

expressed with CYP3A4, CYP2C19 or CYP2D6. This would indicate that co-expressing a second CYP enzyme leads to augmentation of activities of CYP1A2 and CYP2C19. It would also seem that the second CYP is helping to stabilize the compact native state of CYP1A2 and CYP2C19 epitomising the expected effects of 'molecular crowding' (Benton et al, 2012; Despa, 2005; Kuznetsova et al, 2014).

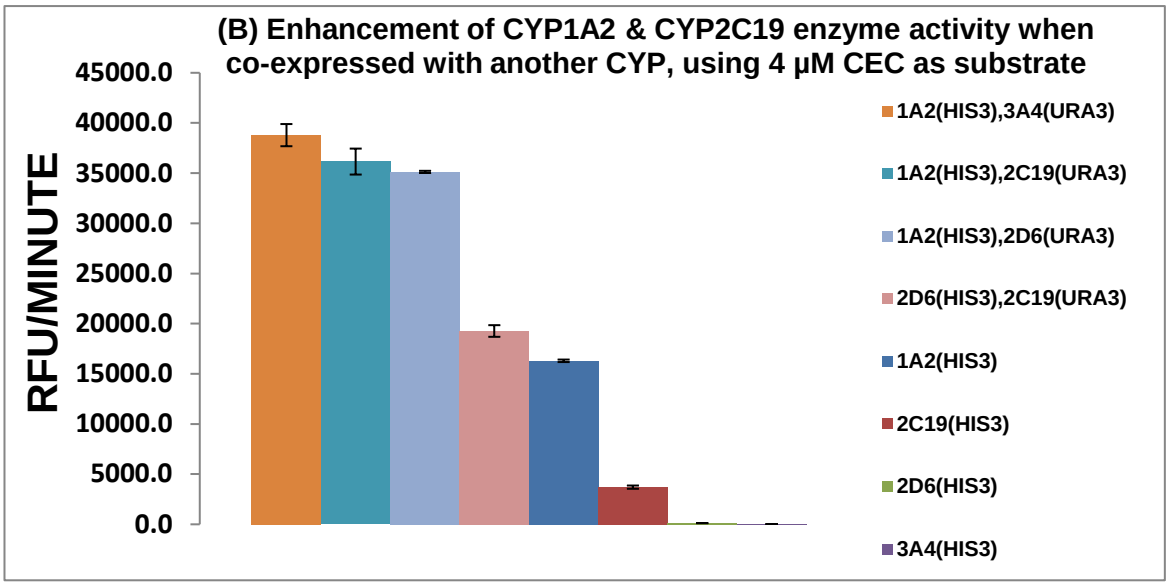


Figure 7.3. The bars mirror the results in Figure 7.2, at the 60 min time point. The activities of CYP1A2 and CYP2C19 are clearly much higher when co-expressed with another CYP than when they are expressed alone. The data represent mean ± S.D. of three independent experiments.

Figure 7.3 corroborates the finding presented in Figure 7.2. The CYP1A2 activity increases more than 2-fold on co-expression of a second CYP whereas the CYP2C19 activity increases 10-20-fold in the presence of another CYP.

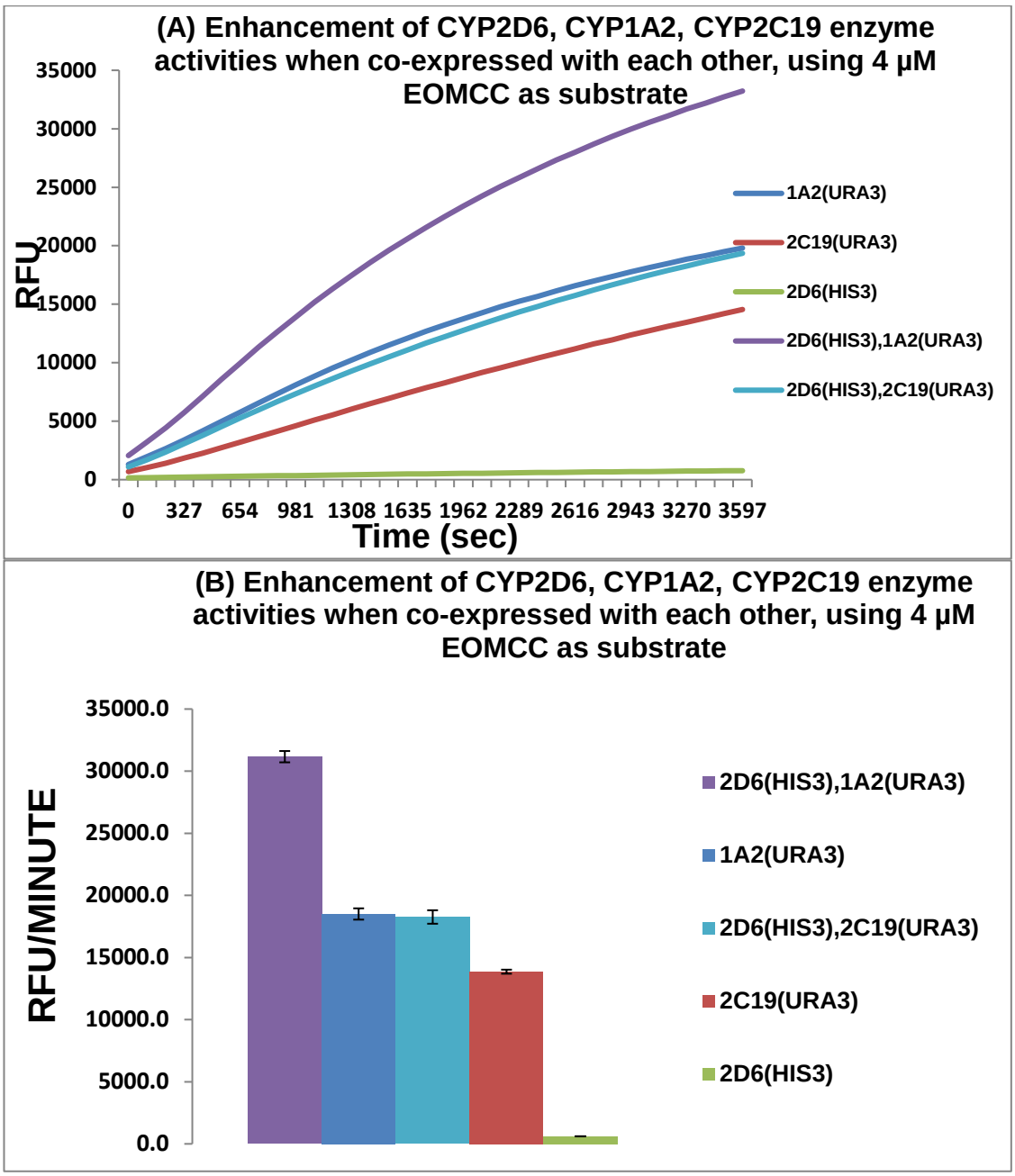


Figure 7.4. EOMCC is a fluorogenic substrate that can measure the activities of CYP1A2, CYP2C19 and CYP2D6. (A) The graphs show the combined activities of these enzymes when co-expressed with each other, in the strain YAB79. The activities are compared with the strains that express single copy *CYP* genes. The graphs represent the average of results obtained from three independent experiments. (B) The bars mirror the results in (A), at the 60 min time point. The activities of the CYP enzymes are clearly much higher when co-expressed with another CYP than when they are expressed alone. The data represent mean ± S.D. of three independent experiments.

Figure 7.4 shows that the co-expressed enzymes have improved activity when compared with the strains that express the single CYP enzymes. These results would again suggest that co-expression of two different CYP enzymes within the same cell stabilizes the activity of each of the enzymes because of the 'crowding effect' (Benton et al, 2012; Despa, 2005; Kuznetsova et al, 2014). For example, the CYP2D6 activity manifested in cells which co-express another CYP (i.e. CYP1A2 and CYP2C19) was seen to be 50 and 30-fold higher than the cells which express CYP2D6 alone.

These results have another separate significance. It has been reported that CYP2D6 protein is degraded in the presence of cytochrome b5, that is, when co-expressed with cytochrome b5 (Hakki et al., 2008; Zehentgruber at al., 2010). From our results, it would seem that this is definitely not the case, at least when CYP2D6 is co-expressed with another CYP enzyme.

The system described in this Section can be used for the simultaneous catalysis of two CYP-mediated reactions. The yeast cell has a robust mechanism of distributing electrons from the haem proteins and the endoplasmic reticulum to allow more than one mono-oxygenation reactions to take place efficiently (De Montellano, 2005; Mike et al., 2005; Guengerich et al., 2001).

7.9 Comparison of CYP1A2, CYP3A4, CYP2C19 and CYP2D6 activities when (a) three of them are co-expressed with (b) the individual enzymes expressed singly

Three different CYP enzymes, from the pool of CYP1A2, CYP3A4, CYP2C19 and CYP2D6, were co-expressed in yeast to see if the crowding effect could still be seen. The results are shown in Figure 7.5.

Results show once again that activities of a CYP enzyme increases when it is expressed together with another CYP enzyme or other CYP enzymes, as was seen before in Section 7.8. The crowding effect, seen in Section 7.8, stabilizes a CYP protein structure, in the presence of another CYP thereby enhancing both CYP enzymes' activity.

Perhaps the crowding effect would be more when three CYP proteins are present, than two CYPs, within the same cohort of cells. The crowding effect did not seem to be further boosted when three CYP enzymes, 2D6, 2C19 and 3A4, were co-expressed in the strain YAB79:: 2D6(HIS3),2C19(URA3),3A4(ADE2) [referred to in Figure 7.5 (A) as 2D6(HIS3),2C19(URA3),3A4(ADE2)] and their expression was monitored using CEC as a fluorogenic substrate. The results were compared with the fluorescence emitted by the strain YAB79:: 2D6(HIS3),2C19(URA3) [referred to in Figure 7.5 (A) as 2D6(HIS3),2C19(URA3)]. Using EOMCC as a fluorogenic substrate, it was clearly seen that co-expression of a third enzyme does not show an increase in expression levels. Stagnancy in the expression levels was seen when CYP3A4 was expressed in a strain which already had the capacity to co-express two enzymes CYP2D6 and CYP2C19. The reactions with both CEC and EOMCC, as substrates, may indicate that saturation may have been reached in the manifestation of the cellular 'crowding effect'.

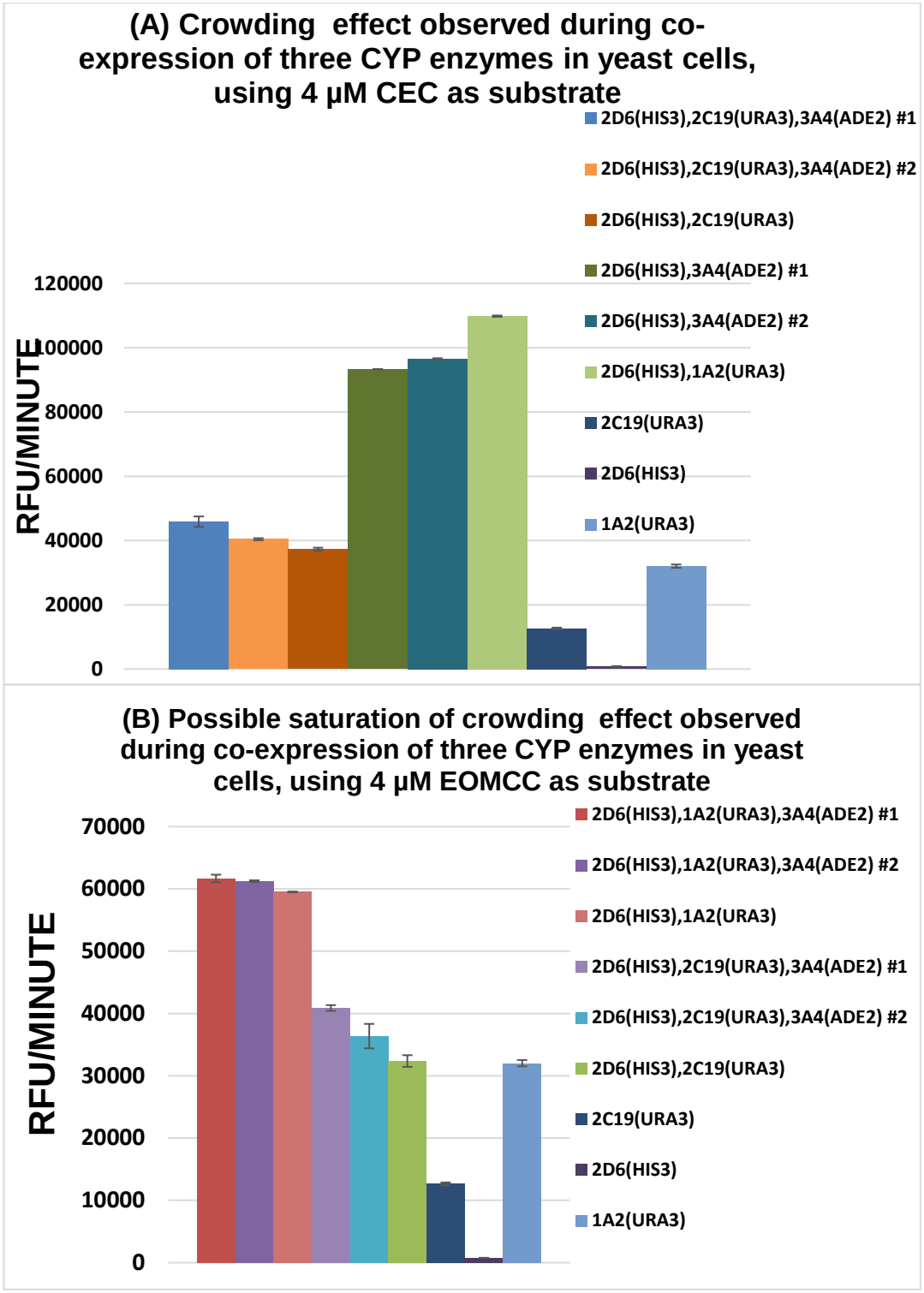


Figure 7.5. The bar plots show the combined activities of CYP1A2, CYP3A4, and CYP2D6 or CYP2C19 enzymes when two or three CYP enzymes are co-expressed. The fluorogenic molecules CEC (A) and EOMCC (B), which were used, can act as substrates for all 4 CYP enzymes. The enzyme activities, from strains which co-express 2 or 3 CYPs were compared with strains that express only one of the CYP enzymes. The data represent mean ± S.D. of three independent experiments.

The results with CEC and EOMCC as substrates suggest that saturation may have set in when the genome of yeast was overcrowded with *CYP* genes. In Figure 7.5 (B), the combined activity of the three CYP enzymes in clones 1 & 2 of the yeast strain [YAB79::2D6(HIS3),1A2(URA3),3A4(ADE2)] referred to in Figure 7.5 (B) as 2D6(HIS3),1A2(URA3),3A4(ADE2)] that co-expresses CYP2D6, CYP1A2 and CYP3A4 (the bars with red and purple colour, respectively) is more or less the same as the activity in the strain YAB79::2D6(HIS3),1A2(URA3) [referred to in the Figure 7.5 (B) as 2D6(HIS3),1A2(URA3)]. This would indicate that the strain that has *three* CYP genes can no longer produce any more amount of CYPs (a reflection of CYP activity) than the strain which has *two* different *CYP* genes.

Similarly, in the two clones of the yeast strain [YAB79::2D6(HIS3),2C19(URA3),3A4(ADE2), referred to in Figure 7.5 (B) as 2D6(HIS3),2C19(URA3),3A4(ADE2)] that co-expresses the three CYP enzymes, 2D6, 2C19 and 3A4, when compared with the strain YAB79::2D6(HIS3),2C19(URA3) [referred to in Figure 7.5 (B) as 2D6(HIS3),2C19(URA3)] that co-expresses only two CYP enzymes, 2D6 and 2C19, it is seen once again that the crowding effect does not permit the combined enzyme activity to move beyond the activity seen in YAB79::2D6(HIS3),2C19(URA3). Possible explanations of these results are that:

1. The binding of substrate may alter the optical absorption spectra of a CYP, thereby making it difficult for the turnover of the substrate probably because of the distances between chromosomal loci, where *CYP* genes are integrated, that could impair turnover (Narasimhulu et al., 1996).

2. The supply of electrons at two different steps in the process of CYP activation may not be functioning in tandem and thus may be having a dissociative effect on the activation of CYPs (Estabrook et al., 1971).

The latter point above involves the first electron being supplied to a substrate-bound oxidized CYP450 so that it can reduce the haem iron at the CYP450 active site. The second electron interacts with the oxygenated P450 to activate the haem-bound oxygen molecule by splitting the O-O bond. This results in the formation of superoxide anion as a by-product of the oxygenation reaction. Superoxide accumulation is toxic to the cells and thereby an inhibitory reaction is triggered. Immediately when the yeast cells sense the toxic environment, they stop production of proteins by trying to preserve themselves. The conclusions from these observations may also have implications on the inhibitory effects of a compound on a CYP enzyme when two or more CYP enzymes are co-expressed within yeast cells.

7.10 Inhibition of a CYP enzyme in the presence of one or two other CYPs which are being co-expressed within yeast cells

The multiple CYP expressing yeast system described above was then tested using known CYP inhibitory compounds to see if it would provide meaningful results in the screening of chemical libraries for CYP inhibition. For studies on the impact of co-expression of multiple *CYP* genes within whole yeast cells, FDA-approved CYP-specific inhibitory compounds were used for screening.

Inhibition of a CYP enzyme mostly involves a reversible reaction. To be able to ascertain the reliability of fluorescent assays in live CYP expressing yeast cells for screening of

CYP inhibitors, several known (FDA-approved) potent inhibitors of CYP2D6, CYP3A4, CYP1A2, and CYP2C19 were used. It has been published that the sensitivity of a CYP enzyme to an inhibitor is dependent on the fluorogenic substrate (Stresser et al., 2000; Wang et al., 2000). In this present study, the following inhibitors were chosen:

(i) Quinidine for CYP2D6,

(ii) Ketoconazole for CYP3A4,

(iii) Furafylline for CYP1A2, and

(iv) Ticlopidine for CYP2C19.

Failure of various CYP inhibitors in drug development is due to the lack of drug-like features and cellular efficacy (Ibidapo Williams et al., 2017). The advantages of expressing CYP enzymes in yeast for drug metabolism studies have been emphasised many years ago by Renaud et al (1993). It is because yeast cells allow correct folding of CYP proteins on the endoplasmic reticular (ER) membranes. Yeast is a simple eukaryote which mimics human cells in many ways. For example, more than 80% of yeast proteins are homologous in their primary sequences to human proteins (Pearson et al., 2013). Thus, CYP-expressing yeast cells could be considered as a convenient and also appropriate tool, which could possibly replace human cells, for early stage drug metabolism studies.

The results for inhibition of the four CYP enzymes, CYP2D6, CYP3A4, CYP1A2 and CYP2C19 expressed within yeast alone, or co-expressed together with one or two other CYPs, after pre-incubation of cells, for 45 min, with compound, are shown in Figures 7.6 to 7.9.

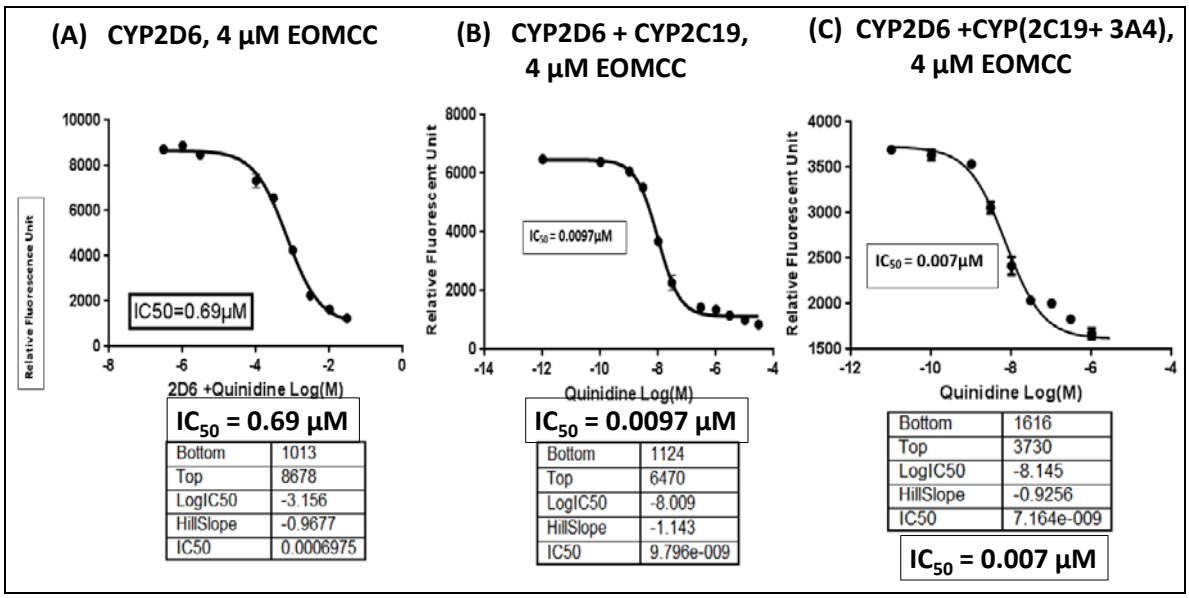


Figure 7.6. Shows the IC50 values of quinidine for inhibition of CYP2D6, in different yeast strains. Inhibition of CYP2D6 in strain that (A) expresses CYP2D6 alone, IC_{50}, 0.69 µM; (B) co-expresses CYP2D6 with CYP2C19, IC_{50}, 0.0097 µM; (C) co-expresses CYP2D6 with CYP2C19 and CYP3A4, IC_{50}, 0.007 µM. The data represent mean ± S.D. of three independent experiments.

The results in Figure 7.6 would indicate that the expression of a second CYP enzyme, CYP2C19, has a great effect on the IC_{50} value of quinidine. The IC_{50} value of quinidine drops from 690 nM (when CYP2D6 is expressed alone) to ~10 nM (when CYP2D6 is co-expressed with CYP2C19). However, when the third enzyme, CYP3A4, is introduced within yeast cells that already have CYP2D6 and CYP2C19, there is practically no further decrease in the IC_{50} value of quinidine which is 7 nM. It is to be noted that the reported IC_{50} value of quinidine after a 'brief' incubation with human hepatocytes, which simultaneously co-express multiple CYP enzymes (albeit at very different levels), is 30 nM ((Peter H. Bui et al. 2008) . Hepatocytes are the predominant cells of the liver and constitute 70-85% of the liver's mass. The difference in the IC_{50} values seen between hepatocytes and cells expressing CYP2D6 with CYP2C19 (30 nM versus 10 nM) could

be explained by the different time periods that were used for pre-incubation of inhibitory compound with CYP2D6 enzyme (45 min versus 'brief' which could be anywhere between 0-10 min). It is known that the time period of pre-incubation affects IC_{50}'s of compounds (Sekiguchi et al., 2009).

This would indicate that expression of a second CYP together with CYP2D6 does have an effect on the inhibitory potential of quinidine. Introduction of a third CYP has no further effect on quinidine's inhibitory capacity. This could be explained by the fact that perhaps expression of a second enzyme together with CYP2D6 already allows the latter to manifest its true 3D geometry whereas addition of another enzyme has no further effect.

The results provide the first indication that the yeast system could be a probable substitute for hepatocytes which are obtained from pools of 200 or more human livers of diverse genetic background. Hepatocytes are also known to lose all CYP activity within 24 h and, therefore, impossible to use for the identification of metabolites of compounds which undergo slow metabolism. Human CYPs being expressed by yeast cells should be able to provide a stable environment, over a week or longer, for the metabolism of compounds that are difficult to study because of their slow metabolism.

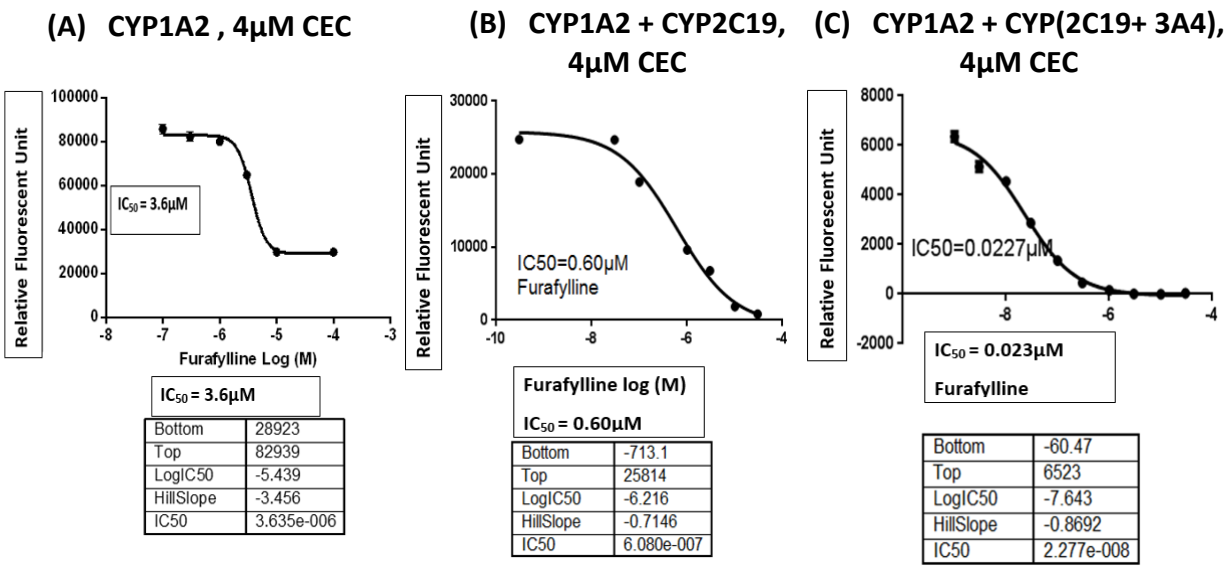


Figure 7.7. Shows the IC50 values of furafylline for inhibition of CYP1A2, in different yeast strains. Inhibition of CYP1A2 in strain that (A) expresses CYP1A2 alone, IC50, 3.63 µM; (B) co-expresses CYP1A2 with CYP2C19, IC50, 0.60 µM; (C) co-expresses CYP1A2 with CYP2C19 and CYP3A4, IC50, 0.0227 µM. The data represent mean ± S.D. of three independent experiments.

The results in Figure 7.7 show that the co-expression of a second and a third enzyme together with CYP1A2 decreases the IC_{50} value of furafylline for inhibition of CYP1A2 from 3.63 µM to 0.60 µM (in the presence of the 2nd CYP2C19 enzyme) to 0.023 µM (in the presence of the 2nd and 3rd enzymes, CYP2C19 and CYP3A4). The percentage inhibition of 1 µM furafylline in hepatocytes, after pre-incubation for 30 min, is reported be 71% (https://tools.thermofisher.com/content/sfs/brochures/ISSX2010InhibitioninHepatocytes.pdf). Pre-incubation of HLMs for 30 min showed an IC_{50} value of 0.586 µM but it is reported that this value could vary depending on the NADPH concentrations in the in vitro assay system; it seems more the NADPH concentration, lesser the IC_{50} value (http://www.cyprotex.com/admepk/in-vitro-metabolism/cytochrome-p450-tdi-ic50-shift). In the cellular system that we have used, the 'crowding effect' (i.e. stabilization of

one protein in the presence of the other) may also play a role in the IC_{50} decreasing from 3.63 µM to 0.60 µM which is the value obtained in human liver microsomes (HLMs). One could also speculate that expression of CYP3A4 which is likely to be produced at high levels within yeast cells, in concert with CYP1A2 and CYP2C19, may be metabolizing (i.e. hydroxylating) furafylline into a more potent inhibitor of CYP1A2.

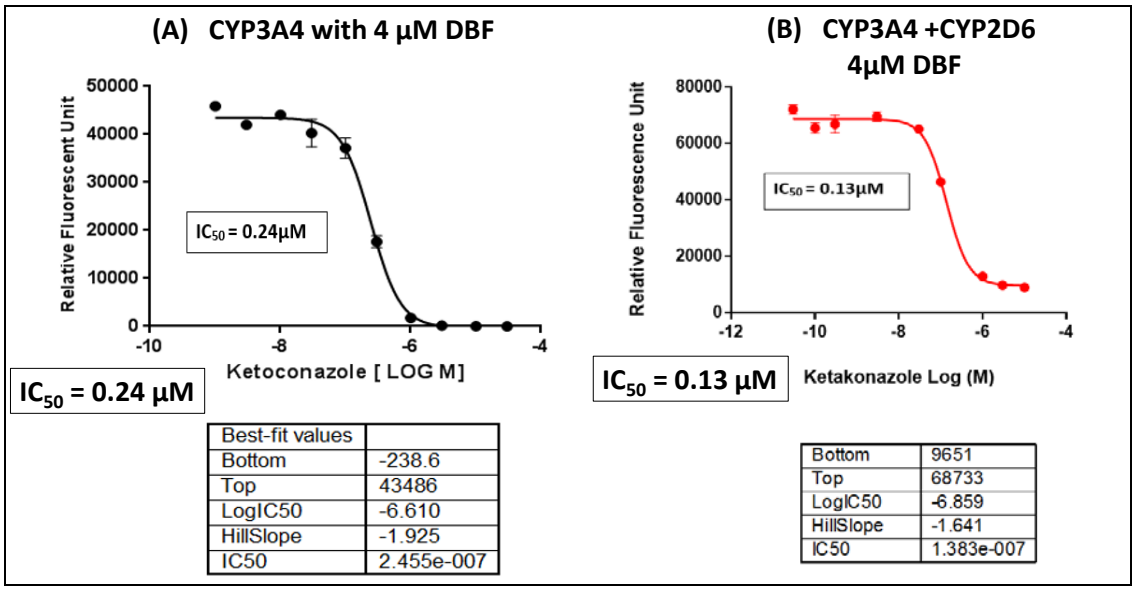


Figure 7.8. Shows the IC_{50} values of ketoconazole for inhibition of CYP3A4, in two different yeast strains. Inhibition of CYP3A4 in strain that (A) expresses CYP3A4 alone, IC_{50}, 0.24 µM; (B) co-expresses CYP3A4 with CYP2D6, IC_{50}, 0.13 µM. The data represent mean ± S.D. of three independent experiments.

The results in Figure 7.8 show that co-expression of CYP2CD6 appreciably decreases the IC_{50} value of ketoconazole from 0.24 µM to 0.13 µM. The reported IC_{50} value of ketoconazole in hepatocytes is 0.14 ± 0.028 (Timothy et al., 2013) which is very close to the value obtained in yeast cells that express CYP3A4 together with CYP2D6. This would again indicate that the yeast system could be a valid substitute for hepatocytes.

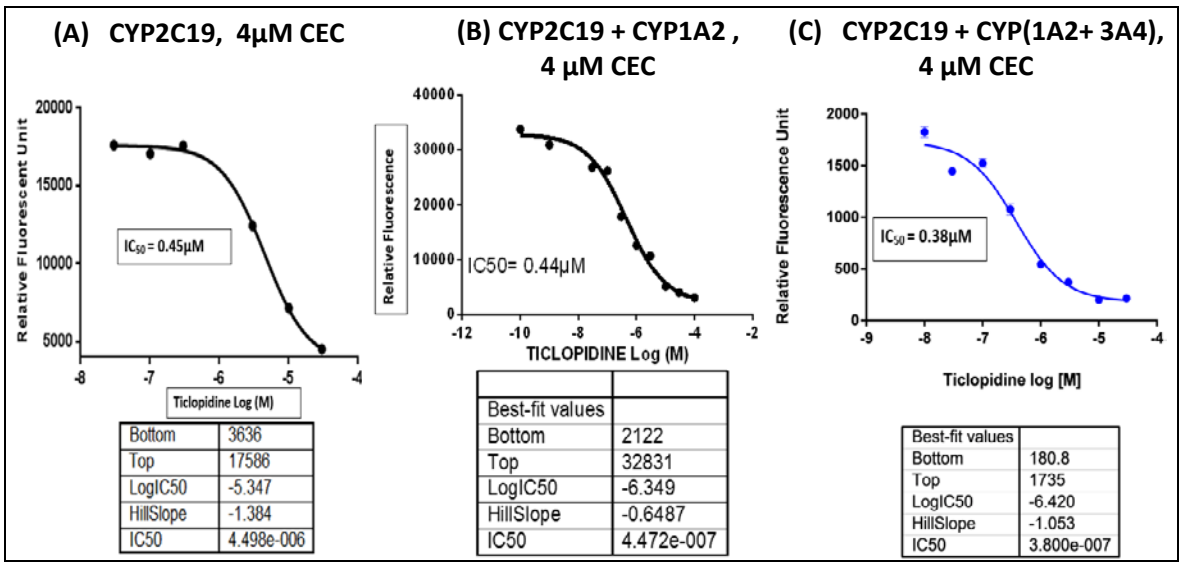


Figure 7.9. Shows the IC50 values of ticlopidine for inhibition of CYP2C19, in different yeast strains. Inhibition of CYP2C19 in strain that (A) expresses CYP2C19 alone, IC50, 0.45 µM; (B) co-expresses CYP2C19 with CYP1A2, IC50, 0.44 µM; (C) co-expresses CYP2C19 with CYP1A2 and CYP3A4, IC50, 0.38 µM. The data represent mean ± S.D. of three independent experiments.

The results in Figure 7.9 show that the IC_{50} value of ticlopidine does not alter too much when two other CYP enzymes, CYP1A2 and CYP3A4 are co-expressed with CYP2C19. This would indicate that CYP co-expression has no effect on CYP2C19 stability. The IC_{50} value of ticlopidine in hepatocytes has been reported to be 0.59 ± 0.2 µM (Stresser et al., 2009). These results suggest once more that human CYP-expressing yeast cells may act as substitute for human hepatocytes.

All the experiments related to IC_{50} determinations of known CYP enzyme inhibitors for a particular CYP enzyme show that the expression of a second and a third human CYP in yeast reveals a new system that could be used in an analogous manner to human hepatocytes. It would be expected that the CYP expressing recombinant yeast cells would provide results as reliable as that obtained from human hepatocytes but with a few added advantages:

(1) Recombinant CYP-expressing yeast cells would produce reproducible results which is very difficult or rather impossible with hepatocytes which are obtained from diverse human beings with very different health status and genetic backgrounds, and

(2) Recombinant CYP-expressing yeast cells could be used to identify the metabolites of compounds, in development, which are difficult or impossible to metabolise with human hepatocytes.

Pan et al (2011) examined the heterologous expression of human CYP2D6 and CYP3A4 in *E. coli* cells. They observed that co-expression increased the level of activities of individual CYPs, after they were isolated as bacterial microsomes, compared to the values in the literature for CYPs that were expressed alone. This study has produced similar results at the cellular level. This was seen when activities of CYP enzymes increased dramatically after co-expression of a second CYP. Introduction of a third CYP shows only slight increase in overall activity. These observations were made across all CYP enzymes that have been expressed until now.

Besides studying potential inhibitory capacity of test compounds, similar cell systems to that which have been described above could also be used to study the possible metabolism of compounds in development. Instead of using individual CYPs which are currently used, whole yeast cells expressing multiple CYPs could routinely be employed to study drug metabolism. This would be analogous to the study that has been reported about tamoxifen (Kwon et al. 2014). It seems that tamoxifen can drastically be detoxified when exposed to a combination of two or three recombinant enzymes simultaneously, for example, CYP1A2, CYP3A4 and CYP2D6 instead of one at a time.

7.11 Conclusions

CYP inhibition assays were successfully set up and performed for CYP1A2, CYP2D6, CYP3A4, and CYP2C19 enzymes. The mean IC_{50} values found for the known inhibitors, furafylline, ketoconazole, ticlopidine, and quinidine, in CYP-expressing yeast cells were consistent with that reported previously in the literature with human hepatocytes (Crespi et al., 1997). Such results with baker's yeast have never been reported before and are, therefore, significant. Moreover, the successful co-expression of more than one human CYP enzyme has also not been reported previously. This shows the importance of the use of yeast biased codons in the expression of human CYP450 enzymes to obtain stable, high-yielding expression of proteins that can be used industrially for larger scale use.

In summary, the present study describes the use of intact whole yeast cells to study the effect of co-production of two or three human CYP enzymes. The expression and inhibition of these co-produced enzymes were studied. The results show yeast has the ability to express multiple human CYPs of choice. Results from inhibition of CYP enzymes expressed within yeast, using FDA-approved CYP-specific inhibitory compounds, show that the yeast system can be used in a similar manner to human hepatocytes. It may follow from these results that real life drug metabolism studies can indeed be performed with human CYP-expressing yeast cells, very early in the drug discovery process. Right now, human hepatocytes are used much later in the pre-clinical/clinical interface. Use of the cheap and reliable yeast system early on could be a boon to the success of future drug discovery programmes.

Chapter 8 Discussion

The aim of my investigation was to devise new systems for expression of cytochrome P450 (CYP) enzymes in baker's yeast, so that they are produced, as 'intracellular membrane bound' proteins, in high yields and activity. It is essential that CYPs bind to intracellular membranes to manifest activity. The goals were to find out the effects on 'activity' and 'yields' of CYP enzymes with the use of (1) human *CYP* genes (~1500 bp in length) which were 'chemically synthesised' using 'yeast-biased codons', and (2) a non-toxic variant of the human P450 reductase gene (ΔhRDM; ~2000 bp in length; Patent WO2007129050A3),. This was to test the concept that 'codon usage' is an important factor in expression of inherently toxic proteins.

8.1 Cloning and constructing of yeast plasmids for expression of 17 human CYP genes (most of which are used for Drug Metabolism studies), synthesized using yeast-biased codons

In Chapter 3, results show the importance of using yeast biased codons in the expression of CYP enzymes. In this Chapter, the advantages of using bakers' yeast as a heterologous expression system were exploited. Yeast has the capability of sheltering CYP enzymes and providing them with a strong anchorage to its intracellular (endoplasmic reticular) membranes. Attempts have been in the past made to use CYP-expressing yeast cells to study drug metabolism in vitro but not at all effectively (Oeda et al., 1985).

The composition of phospholipids, sphingolipids, and other sterols in the membranes provide a valuable influence on the activities of the protein associated or embedded in the lipid bilayer (Rest et al., 1995; Ali et al., 2006). Hence, binding to the yeast endoplasmic reticular membranes must have an influence on CYP enzyme activities. All activities of CYP enzymes, expressed from synthetic *CYP* genes (i.e. synthesized using yeast biased codons) have shown high activity when expressed in yeast form episomal plasmids. The results were compared head-to-head with the enzymes expressed from 'native' *CYP* genes obtained from a human liver cDNA library. It was seen that the amount of proteins produced by the synthetic genes were far more than that produced by native genes. For particular CYPs, the presence of cytochrome b5 enhanced the levels of CYP proteins that were produced.

Customarily, CYP1A2 is expressed in the absence of cytochrome b5. It has been reported that cytochrome b5 has no effect on CYP1A2 activity or expression level when it was co-expressed (Zhang et al., 2015; Duarte et al., 2005). However, during this study, when cytochrome b5 was co-expressed with CYP1A2 (i.e. from strain YAB97::CYP1A2), a higher activity was observed when compared with the strain YY7::CYP1A2, that expressed CYP1A2 in the absence of cytochrome b5 (Figure 4.37; Chapter 4). This provides room for further studies on CYP1A2 expression to see if with the use of further chromosomal integrations if one could construct a strain that would express even higher levels of CYP1A2. This would also provide an insight into the stability of CYP enzymes, in general, after their overexpression (see Chapters 4 & 5).

In addition, a similar study was carried out with the two CYPD2D6 variants, CYP2D6(Met^{374}) and CYP2D6(Val^{374}) where the variant enzymes were expressed in the

absence or presence of cytochrome b5. Results show that cytochrome b5 has a deleterious effect on both CYP2D6 variants (Figures 4.45 and 4.52; Chapter 4).

It has been reported that the residues at position 374 of CYP2D6's primary sequence, Val and Met, play at an important part in the active site of the enzyme. It has been proposed that the residues at this position hep in binding substrates and inhibitors of CYP2D6. It was suggested that a mutation at the DNA level that can cause a change from in amino acid from Met to Val can form a hydrophobic cleft in the loop between the K helix and β l-4 (Ellis et al, 2000), thus altering the rate of enzyme-substrate reaction and also affecting CYP2D6's stereo-selectivity in the metabolism of the drug, metoprolol. In the same report, however, it was stated that the kinetics of metabolism of other drugs are totally unaffected by this change. The report also reiterated that Val^{374} is the actual residue in the wild-type protein which is in contrast to another report that claims that Met is the residue at position 374 of the wild-type protein (Rowland et al., 2006).

Besides expressing the above two variants, Met^{374} and Val^{374}, three CYP2D6 SNPs (mutants, *2, *10 and *39), were also 'synthesised using yeast biased codons. Western blotting, after expression of the SNP bearing gene constructs in yeast, shows that codon bias helps in providing much more CYP2D6 protein for the variants that bear the SNPs when compared with the enzyme expressed from the native *CYP2D6* gene, isolated form a human liver cDNA library (Figure 3.45; Chapter 3).

Yu et al (2003) have reported the contribution of CYP2D6 polymorphism and has described the pharmacokinetic variability of CYP2D6 SNPs in humans. This report suggests that CYP2D6(Met^{374}) (i.e. 2D6-2) possesses similar affinity towards the fluorogenic substrate as the three enzymes expressed from genes that bear the SNPs. used

than any other reported CYP2D6. Chromosomal integration of gene coding for the CYP2D6(Met^{374}) (i.e. 2D6-2) and CYP2D6(Val^{374}) (i.e. 2D6-1) variants has led us to further quantify expression levels and CYP2D6 enzyme activities (Chapter 5).

The enzyme aromatase (CYP19A1) is responsible for the conversion of testosterone to estradiol in many tissues. Aberrant expression of CYP19A1 is one of the primary causes of breast cancer in postmenopausal women (Nadine et al., 2016; Boon et al., 2010). CYP19A1 enzyme is also misregulated in prostate and colorectal cancer (Travis et al., 2009; Slattery et al., 2011. The expression level of CYP19A1 and its enzyme kinetic activity was investigated during this study. CYP19A1 shows good level of expression in yeast which was independent of the presence/absence of cytochrome b5 (Figure 3.84; Chapter 3). Previously published reports did indicate that presence of cytochrome b5 was essential for CYP19A1 expression (Akhtar et al., 2015).

Although CYP2C8, CYP2C9 and CYP2C19 play important role in drugs metabolism, factors that mediate and control the activity of the CYP2C subfamily of enzymes are largely not well defined. This study has utilised the importance of yeast biased codons to clone these CYP genes in baker's yeast. It was observed that expression levels of CYP2C8, CYP2C9 and CYP2C19 were >10, 0.5 and 5.5 fold higher, respectively, than that observed in the strains that express the 'native' genes cloned from a human liver cDNA library (Figures 3.59, 3.14, 3.19; Chapter 3). Microsomal CYP2C9 and CYP2C19 enzymes were isolated from yeast cells. Results showed that microsomal enzymes, isolated from cells which contained integrated copies of *CYP* genes, could be prepared in very high yields and activities compared to what would be possible using strains that contained *CYP* gene-bearing episomal plasmids (Figures 5.20/5.21/5.29 and

5.19/5.20/5.30; Chapter 5). Past studies have used cultured human hepatocytes in the study of expression of CYP2C enzymes (CYP2C8, CYP2C9, CYP2C18 and CYP2C19). The results have shown that deletion of cytochrome b5 is responsible for the reduction in the metabolism of drugs. The presence of cytochrome b5 is required for increased expression of these CYP enzymes (Henderson et al., 2014). In this study, cytochrome b5 has been co-expressed with CYP2C8, CYP2C9 and CYP2C19 enzymes. We also see that, in the presence of cytochrome b5, expression levels increase dramatically, particularly in the case of CYP2C9 and CYP2C19. Contrary to this observation, cytochrome b5 did not have any influence on the levels of CYP2C18 enzyme expressed from the gene synthesized with yeast biased codons.

The human CYP2B6 enzyme participates in the catalysis of several clinically important drugs and xenobiotics (Mo et al, 2009). It has been reported that polymorphism affects the expression levels of CYP2B6 (Lang e al., 2001). Zhanger et al, (2007) confirmed that there are 29 polymorphic alleles of CYP2B6. In this study, the human *CYP2B6* gene has been synthesized with yeast biased codons and expressed in baker's yeast. The results show that levels of expression in the presence of cytochrome b5 was high (Figure 3.24; Chapter 3), although this is in contrast to what has been reported before (Xie et al., 2003). This enzyme is involved in the clearance of the drug efavirenz (Hass et al., 2004; Tsuchiya et al., 2004; Zanger et al., 2007).

The CYP isozyme CYP2E1 is involved in the metabolism of alcohol, aldehydes and various carcinogens. Upregulation of CYP2E1 has been linked to diseases like diabetes, obesity, cancer, liver cirrhosis and non-alcoholic hepatic steatosis (Sumner et al., 1999).. The most interesting role of the enzyme is its ability to oxidize ethanol into reactive

products, acetaldehyde and 1-hydroxylethyl radical which is a reactive oxygen species (ROS). Production of ROS leads to oxidative stress. Large fractions of CYP2E1 are located in the endoplasmic reticulum (Bansal et al., 2010). In this study, I have assessed the expression in baker's yeast of human CYP2E1 enzyme using its gene (*CYP2E1_yc*) synthesized with yeast biased codons. The expression of CYP2E1 was higher when compared with that obtained from 'native' *CYP2E1* gene isolated from a human liver cDNA library (Figure 3.49; Chapter 3). The initial results obtained were further optimised by integrating the *CYP2E1_yc* gene expression cassette on to the yeast genome at the *HIS3* chromosomal locus (Figure 4.87, Chapter 4; Figure 5.33, Chapter 5).

It has been reported that cytochrome b5 has no effect on the activity of the human CYP2A6 enzyme. The turnover of the enzyme in the presence or absence of cytochrome b5 did not show any difference (Duarte et al., 2005). In this report, the *CYP2A6_yc* gene, synthesised using yeast biased codons, has been used for expression in yeast in the presence of cytochrome b5. The expression level was much higher when compared with the one obtained from native gene (Figure 3.54; Chapter 3). A Western blot confirmed the initial observations (Figure 3.55; Chapter 5). The results again showed that expression of a gene with yeast biased codons influences expression levels of a CYP enzyme.

It has been reported that inhibitors of CYP1 (CYP1A1 and CYP1B1) enzymes may play a vital role in the prevention of cancer (Androutsopouos et al, 2009) and overcoming chemo-resistance to anticancer drugs (Williams et al., 2017) in human cells that overexpress CYP1A1 or CYP1B1. Also in Chapter 6 of this study, CYP1A1 mediated more than 90% conversion of chrysin to baicalein. Whole yeast cells stably expressing CYP1A1 was used for this conversion (Williams et al., 2017). The use of yeast biased

codons has provided an avenue to optimise the expression of CYP1A1 and CYP1B1 to produce high yields of these enzymes. When protein expression was compared with strains expressing native genes, isolated from a human liver cDNA library, results showed that there was a lot higher expression in strains containing genes synthesised with yeast biased codons (Figures 3.64, 3.69; Chapter 3).

CYP2J2 is also known as unsaturated fatty acid epoxygenase. It has attracted much attention to its metabolism of arachidonic acid (AA) (Wu et al, 1996) to four different epoxyeicosatrienoic acid metabolites (EETs) (Xu et al, 2013; Morrison et al., 1981). N-terminal truncation of CYP2J2 was reported to be the only way to express this enzyme (Park et al, 2014). But in this study, the synthetic *CYP2J2* gene with yeast biased codons when expressed in baker's yeast has shown good expression (Figure 3.89; Chapter 3). Results from a Western blot show that the amount of CYP2J2 protein obtained is more in the presence of cytochrome b5 result than in its absence Figure 3.90; Chapter 3).

CYP4F3A and CYP4F3B are generated via the selection of different transcription start sites (Christmas et al. 1999) resulting from an alternative splicing of exons. CYP4F3A mediates the production of human eosinophil (Kikuta et al., 2007). In the presence of cytochrome b5, there is a good level of expression of the CYP4F3A enzyme (Figure 3.74; Chapter 3). Microsomal enzyme has been isolated from yeast. Our results show that the activity of the yeast-produced enzyme is considerably better than the only commercially available enzyme from Corning-Gentest (Figure 5.34; Chapter 5).

The CYP17A1 enzyme was expressed from a *CYP17A1_yc* gene, synthesised with yeast biased codons, in the presence and absence of cytochrome b5. Level of expression in the presence of cytochrome b5 was much higher than in its absence (Figure 3.79; Chapter 3).

Western blot analysis confirmed that the expression level was higher in the presence of cytochrome b5 in all the CYPs (Figure 3.80; Chapter 3). The membrane haem protein cytochrome b5 can enhance or inhibit the catalysis of CYP450 proteins but this depends on the specific CYP450, the substrate, and reaction conditions. But the structural basis for enhancement or inhibition is still unclear (Estrada et al., 2013, 2105). CYP17A1 is a critical enzyme in steroidogenesis, and acts between the points of glucocorticoid synthesis and androgen synthesis (Duggal *et al*., 2016). Cytochrome b5 is known to be the chief regulator of CYP17A1 reactivity in vivo (Auchus et al., 1998). Like the observations we have during this study, cytochrome b5 is known to enhance the CYP17A1 mediated lyase reactions 5 to 10 fold (Gurvey et al., 2001). Although the specific role of cytochrome b5 is not yet clear, but in a study recently published it was deduced that cytochrome b5 is likely to play the role of a second electron donor (Duggal et al., 2016). The results obtained in this study confirm that expression of CYP17A1 requires co-expression of cytochrome b5 for production of high levels of protein.

Overall, all expressions with *CYP* genes, synthesised with yeast biased codons have shown a great improvement in the levels of CYP proteins over those produced from the native genes isolated from a human liver cDNA library. The use of yeast biased codons has produced a platform for the experiments performed in the following Chapters.

In Chapter 4, the synthetic *CYP_yc* genes, synthesized with yeast biased codons, have been used for integration into the yeast genome with the aim of constructing stable yeast strains (cell lines) that can be grown in non-selective, highly nutritious YPD medium.

8.2 Construction of yeast strains that would stably produce cytochrome P450 (CYP) enzymes from genomic copies of the CYP genes

We have already seen the role yeast biased codons play to enhance the activity of the seventeen CYP enzymes that have been described in this study (Chapter 3). All experiments in Chapter 3 were performed with yeast strains containing episomal plasmids. Maintaining these plasmids (i.e. extra-chromosomal entities) within yeast cells requires a selective medium which is, firstly, not nutritious enough to provide healthy growth and, secondly, cannot maintain the plasmid over many generations. Minimal media growth is seldom prolonged beyond 48 h which equates to 32 generations of growth (yeast cell division occurs every 90 min).

In this context, it was thought that if the *CYP_yc* gene expression cassettes (containing the *CYP_yc* gene sandwiched between the *ADH2* promoter and the *SUC2* terminator) were to be integrated into the yeast genome, the problems related to loss of genetic information from the cells would not exist. Hence, all *CYP_yc* expression cassettes were integrated into specific chromosomal loci afforded by the auxotrophic markers of the yeast strain (i.e. BC300) that had been chosen for expression of the CYP enzymes. The loci available for integration (after having integrated a mutant human CPR and, in some cases, cytochrome b5) were at the locations where the *ADE2*, *HIS3* and *URA3* genes lay on particular chromosomes of the yeast genome.

It was also thought that use of the *ADH2* promoter would be of great help. To the best of our knowledge, it is the only yeast promoter that naturally allows a slow and gradual

induction of expression of foreign proteins when cells are grown in glucose, the beast carbon source for growth of all living cells.

8.3 Human CYP enzyme expression from a single chromosomal location of the yeast genome

The yeast integrative plasmids, containing a CYP gene expression cassette, used the *ADE2*, *HIS3*, or *URA3* genes as a yeast selection marker. The integrative plasmids were linearized within these marker genes. The linear DNA is flanked at either end by the 5'-end and the 3'-end of the marker genes. These flanking regions allow homologous recombination at the chromosomal sites of the yeast genome which bears the dysfunctional *ade2*, *his3* and *ura3* genes. If homologous recombination were to occur, a *CYP_yc* gene expression cassette introduced into the yeast genome would allow the cells to become *ADE2*, *HIS3*, or *URA3* (i.e. the genes would become functional again), depending on the integration plasmid used. This would allow initial selection of cells that contain an integrated *CYP_yc* expression cassette in minimal SD medium that lacks adenine, histidine or uracil, again depending on the integration plasmid used. Once the integration is confirmed in SD medium, the cells harbouring an integrated gene expression cassette or gene expression cassettes could be propagated indefinitely, without any selection, in full complete YPD medium.

Integration of a single copy of various *CYP_yc* gene expression cassettes and further biochemical analysis of the expressed enzymes are described in Chapter 4, Sections 4.6 to 4.16.

Yeast strains for the following human CYP enzymes were constructed with a *CYP_yc* gene copy at the *HIS3* or *URA3* locus: 1A2, 3A4 3A5, 2D6-1(Val^{374}), 2D6-2(Met^{374}), 2C9, 2C19, 2E1 and 4F3A. In the case of 3A4, the *CYP3A4_yc* gene was also integrated at the *ADE2* locus.

Cellular assays were developed from scratch to assay each of the human CYP enzymes that were expressed in baker's yeast. This had to be done since such type of cells have never been reported before. Fluorogenic substrates, which are non-fluorescent to start off with but which fluoresce when a product is formed, were used to set up the enzyme assays. These substrates were also very helpful in selecting high producing clones from each strain. Often 50 clones were screened to obtain a high CYP enzyme producer which could be later used for (a) isolation of microsomal enzyme and (b) biotransformation reactions.

Results with all CYP enzymes tested show that chromosomal integrations of a *CYP_yc* gene provides much higher levels of a CYP than a strain containing *CYP_yc* gene bearing episomal plasmid, even after 18 h of growth which was quite unexpected. Minimal plasmid loss should occur during an 18 h period of yeast cell growth. The results indicate that chromosomal integrations provide stability to the foreign genetic information which yeast cells propagate during cell division.

In general, it was observed that the *HIS3* chromosomal location provided the highest levels of a functional CYP enzyme. As discussed in Chapter 4, this may be due to the neighbouring proteins within chromosome XV, where the HIS3 gene lies, that may be acting in *trans* to regulate the expression of *CYP* genes under the control of the inducible *ADH2* promoter.

8.4 Expression of a CYP enzyme transcribed simultaneously from two or three gene expression cassettes, located at different chromosomal loci

The importance of a chromosomal integrated copy of a human *CYP* gene expression cassette has been described in the above Sections. The development of one copy yeast strains overcomes the problem of stability encountered when using episomal plasmids. To explore further, a second copy of a *CYP* gene was added to an existing copy contained within yeast strains. It may be logical to assume that a second copy will provide better levels of functional CYP enzymes than from a single copy. However, CYP enzymes are inherently toxic because of their redox properties allowing them to generate high levels of ROS during overexpression. Since already it has been seen that a chromosomal integrated copy produces a lot more CYP enzyme than from an episomal plasmid, even at 18 h of growth, it may have been unrealistic to expect that there would be more CYP protein produced if expression occurred simultaneously from two separate chromosomal locations.

The *HIS3* gene belongs to yeast chromosome XV and the *URA3* gene is an integral part of chromosome V. It may be possible that transcription of two independent copies of a *CYP_yc* gene from two different chromosomal locations may be able to enhance levels of proteins made through ‘neighbouring effect’ or ‘crowding effect’ (Benton et al., 2012 ; Despa, 2005).

The results in Chapter 5 show that a strain bearing two copies of a CYP gene does produce more CYP enzyme than the strain containing a single copy.

The microsomal CYP enzymes were isolated from strains containing (a) two integrated copies of the *CYP_yc* gene and (b) an episomal plasmid which encodes the *CYP_yc* gene. The amounts of functional CYP enzymes were measured via carbon monoxide (CO) difference spectroscopy as was described in Chapter 2. It involves binding of CO to CYP450 to measure the amounts of CYP450 produced. The results show that amount of an enzyme produced from 400 ml of cells, cultured from strains containing two integrated copies of a *CYP_yc* gene, are a lot more than the enzyme produced from the same number of cells containing a *CYP_yc* gene bearing episomal plasmid. The Western blots show a clear difference in the amounts of CYP enzymes produced within the two types of cells.

The IC_{50} values of know inhibitory compounds, using the Sacchrosomes (yeast derived microsomal CYP enzymes) show that the values obtained are close to those published for human liver microsomes (HLMs) indicating that the CYP-Sacchrosomes possess active sites that are similar to the native enzymes produced in human liver cells. This indicates that instead of depending on expensive HLMs, Sacchrosomes can reliably be use for rapid screening of CYP inhibitory compounds.

8.5 Roles of yeast bias codon synthesised recombinant CYPs in biocatalysis

In Chapter 6, biotransformation reactions were performed using whole yeast cells. Three CYP enzymes, CYP1A1, CYP2D6, and CYP3A4, contained within whole yeast cells have been used for the biocatalysis of interesting substrates, as a proof of principle that whole yeast cells can be used for efficient biotransformation.

CYP1A1 was used for the conversion of chrysin to baicalein. More than 90% of chrysin was converted to baicalein after 144 hrs of incubation with whole cells. The conversion involved selective hydroxylation at the C6 position of chrysin. Molecular modelling (PDB ID: 418V) has explained this selectivity.

Whole yeast cells expressing CYP2D6 variants have been used to study the metabolism of codeine to morphine. A novel CYP2D6 variant (P34S/A122V/S486T) has been identified serendipitously. It converted > 60% of codeine to morphine whereas the other two variants (Met^{374} and Val^{374}), both of which have often been referred to as wild type, convert < 30% of codeine to morphine. The Met^{374} and Val^{374} variants produce a lot more nor-codeine through *N*-demethylation than the other variant which predominantly catalyses *O*-demethylation to morphine.

Whole yeast cells expressing CYP3A4 have been used to follow the biotransformation of a medicine in phase II/III clinical trials. It had been disclosed that all commercially available CYP3A4-based test systems were able to convert < 10% of the compound AZD-2014 to its metabolites. We have shown that approximately 80% of AZD-2014 was converted to metabolites X and Y using CYP3A4 expressing whole yeast cells.

8.6 Crowding effect through the co-expression different CYP enzymes within whole yeast cells

The activities of CYP1A2, CYP2D6 and CYP3A4 were analysed after c-expression, using fluorogenic substrates CEC and EOMCC. The results show that the CYP2C19 activity is greatly enhanced when co-expressed with CYP2D6. In parallel, CYP1A2 activity is remarkably improved when it is co-expressed with CYP3A4, CYP2C19 or CYP2D6. This would indicate that co-expressing a second CYP enzyme leads to stabilization of the 3D structure, thereby activities, of CYP1A2 and CYP2C19. This may be an indication that 'molecular crowding 'or 'neighbouring effect' is playing a role in stabilisation.

The CYP inhibition assays were successfully set up and performed for CYP1A2, CYP2D6 and CYP3A4 enzymes. The aim was to determine whether there are any differences in IC_{50} values of the co-expressed CYPs in comparison to the values reported in hepatocytes. The mean IC_{50} values found for the known inhibitors, furafylline, quinidine and ketoconazole were consistent with that reported previously (Crespi et al. 1997). These inhibition studies demonstrated that CYP-expressing whole yeast cells can be used to quantify the inhibition of CYPs by chemical compounds.

8.7 Summary

In summary, we have devised various yeast systems that produce functionally active human CYP enzymes. The CYP genes that were used were chemically synthesised using yeast biased codons. It has been shown clearly in the early part of the thesis that these biased codons definitely facilitate expression of human CYP enzymes in yeast. The use

of yeast biased codons has allowed us to achieve our goal of improving expression levels for all human CYPs that have been studied.

The key findings of this thesis are listed below:

1. Expression of high levels of CYP proteins from episomal plasmids encoding genes with yeast biased codons. It allowed us to answer the question whether chemically 'synthesized' *CYP* genes produce active CYP enzymes that are as good as the enzymes produced from the 'native' genes. It was found that genes with yeast biased codons produce a lot more protein than the native genes.

2. *CYP* genes integrated on a chromosomal locus have a huge effect on the yields and activities of all CYP enzymes produced. The *HIS3* locus was the best for expression.

3. The integration of two or three *CYP* genes produced higher yields than the single copy integrated strain. This allowed us to create improved systems that may allow convenient (a) drug metabolism studies and (b) bioorganic synthesis of high-value chemicals via biotransformation.

4. Human CYP microsomal enzymes isolated from yeast were far superior to commercially available CYP microsomal enzymes from the three leading manufacturers of these enzymes which are isolated from insect and bacterial cells.

Furthermore, multiple enzymes co-expressed within the same yeast cell produced much higher yield when compared with the individual CYP enzymes expressed from yeast cells. This allowed us to create a system which on further development could possibly be used as a substitute of human hepatocytes for drug inhibition and metabolism studies.

8.8 Future prospects

In this study, we have established the role of the yeast biased codons and integration of genes containing these codons into the genome of yeast cells provides proteins with high yield and activity. Nonetheless, there is more that needs to be done to establish the reasons why cytochrome b5 is more selective towards certain CYP enzymes. If we can change the locus of cytochrome b5 in the parent yeast, maybe we could establish this reason. Also, in 1999, Zhao and colleagues studied the crystal structure of the FMN binding domain of human cytochrome P450 reductase (CPR). Their findings indicate that the FMN domain has an unusual surface charge distribution, leading to a very strong dipole which may be involved in docking a CYP into place for electron transfer (Zhao et al. 1999). This could be a clue for future optimisation of production of CYP450 enzymes in large scale. If we can create an artificial electron donor in some of the strains that are already producing CYP450, maybe the time scale for production could be cut down and a higher yield would be achieved. As we understand, all CYPs have different sites in their binding surface for receiving of necessary electrons from NADPH. This would be achieved using methods that will mimic the surface of cytochrome b5. This will prevent competition from cytochrome P450 reductase for binding to the site of some CYP450 enzymes which happen to be inactive when co-expressed with cytochrome b5.

CHAPTER 9 REFERENCE

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